

**Sixth Edition**

**FUNDAMENTALS OF  
EXPERIMENTAL  
PHARMACOLOGY**

**M. N. GHOSH**

**HILTON & COMPANY**

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Sixth Edition

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## PREFACE

It is more than four decades since the first publication of the book in the year 1971 with an introduction by late Prof. H. O. Schild, F.R.S, M.D., Ph.D., DSc., Professor of Pharmacology, University College, University of London, which is being reproduced here for the knowledge of the present generation of young scientists. During the intervening period, there have been remarkable developments in the various fields of biomedical sciences. In order to keep pace with these developments, there had to be a gradual shift in the experimental studies from the whole animals and organs to the cellular and subcellular levels. It is also clear now that experimental pharmacology cannot remain bound to a water tight compartment, but has to be intermingled with other scientific disciplines to bring about fruitful results. This prompted inclusion of chapters like *Alternatives to Animal Experiments* and *Molecular Cell Biology* in the previous two editions of the book. In the present edition, there has been further extension by incorporating chapters like *The Cell Line Studies* and *Nanotechnology in Biomedical Science*. Besides these, there have been some additions in the chapters on *Pharmacology of Receptors, Adrenergic Receptors, Opioid and Cannabinoid Receptors*, and *Angiotensin, Prostaglandin and Nitric Oxide*. Since a lot of research papers are being published on indigenous plant materials both in Indian and foreign journals, it was thought to be useful to include a chapter on *Methods of Plant Extraction*. I am extremely thankful to the authors for their valuable contributions in the present edition of the book. The format of the book as well as the cover design have completely been changed giving it a new look for which Mr. Sanjoy Ghosh and Mr. Sandip Ghosh of Hilton & Company deserve compliments.

Kolkata, India  
December, 2014

**M.N.Ghosh**

## **PREFACE TO THE FIRST EDITION**

Just as medicine cannot be taught or learnt without ward clinics so also pharmacology without animal experimentation. Today's pharmacology is much different from yesterday's materia medica. The former not only encompasses the general definition 'science of drugs' but also the meaning in a more restricted sense, that is, 'action of drugs on living organisms'.

Today experimental pharmacology forms an integral part of teaching as it helps in making clear and interesting what is being taught in didactic lectures. A number of excellent books on experimental pharmacology are already available for research workers. Most of them, however, are fairly advance in nature and in scope demanding a sound knowledge of fundamental principles on the part of the readers. The purpose of the present title is to provide such fundamental knowledge on subjects ranging from laboratory animals and methodology to statistical analysis and interpretation of results. Such elementary but essential points, for example, how to adjust the magnification or tension of a writing lever, form the basis of this book. Attempts have been made to include as much relevant information as possible and to illustrate points with suitable examples thus making the book comprehensive but taking care at the same time not to make it unwieldy.

If this book has been of any help to the postgraduates, research workers and junior teachers in the training for a higher degree, in the planning of research, or in organizing demonstrations and exercises in experimental pharmacology, it would have served the purpose for which it was written.

August, 1970

M. N. GHOSH

## INTRODUCTION

My Friend and old colleague Dr. M. N. Ghosh has sent me this book and I am happy to write a brief introduction for it. Its purpose is to provide a concise account of some of the methods and general principles which form the basis of pharmacological experimentation. Books of this sort are rare and by no means easy to write because pharmacology, more than most natural sciences, lacks a well established theoretical foundation. Yet, without theory, a subject, however rich in facts and observation, cannot truly advance and will eventually get bogged down. It is therefore important that the limited amount of general pharmacology available should be considered and mastered by students.

One of the useful aspects of the book is that it contains a number of worked-out examples which will help to translate theory into practice. I believe that it will fulfill a need and occupy an important niche in the development of experimental pharmacology in India.

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# Common Laboratory Animals

## Animal Experimentation vis-à-vis Computational Simulation

The most recent development in experimental pharmacology is the sophisticated computational simulations of animal experiments, both *in vitro* and *in vivo*, referred to as *in silico* test. It is so named because of its development in silicon on a computer chip. It is a simplified ecological model that reduces a lot of animal testing, and considered to be more predictable regarding the success of the developing drug in clinical trial. Despite these advantages, the animal experimentation cannot be done away with completely, and the drug in question has to be administered at some stage or the other, into an animal before it can be passed on safely to humans.

## Care and Handling of the Laboratory Animals

The handling of the laboratory animals involves two most important responsibilities on the part of the experimenter. First, the animal is handled with utmost care so that it does not suffer any pain, and secondly, a due regard is paid towards the health and well being of the animal colony. Even when they are killed at the end of the experiment, it should be done by a humane method, i.e. *euthanasia*, which means painless killing.

The Indian National Science Academy (INSA)\* provides the following guidelines to be followed by all research institutions in the country engaged in animal experimentation.

1. Housing, care, breeding and maintenance of experimental animals to keep them in physical comfort and good health, and to permit them to grow, reproduce and behave normally.
2. Sources of experimental animals of known genetic, health and nutritional status.
3. Development of training facilities for scientists, technicians and other supportive staff for the care of animals and their use in experiments.
4. Acceptable experimental techniques and procedures for anaesthesia and euthanasia.
5. Developing alternate *in vitro* systems to replace animal experiments.
6. The constitution of institutional ethics committees, their functions and the legal and ethical obligations to ensure minimal and ethical use of animals.

\* Guidelines for Care and Use of Animals in Scientific Research, Published by Indian National Science Academy, New Delhi, 1992 & 2000.

Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA)\* has also set guidelines for laboratory animal facility. The goal of these guidelines is to promote the humane care of animals used in biomedical and behavioral research and testing with the basic objective of providing specifications that will enhance animal well being, and quality in the pursuit of advancement of biological knowledge that is relevant to humans and animals.

### Breeding

Most of the common laboratory animals such as mice, rats, guinea pigs and rabbits can be bred and reared under ideal conditions to provide a reliable supply of uniform and healthy animals for the purpose of experimentation. Dogs and cats, however, can be obtained from outside sources as and when required, but it is always better to keep them under quarantine for a few weeks before they are utilized.

### Breeding Types

*Inbred strains.* The primary purpose of inbreeding is to reduce the number of individuals that are heterozygous for any one gene pair, and to increase the number that are homozygous for one or the other member of the gene pair. *Coefficient of inbreeding* is the degree of inbreeding as measured by the reduction in the number of heterozygotes and increase in the number of homozygotes. A strain is regarded as *inbred* when it has been mated full brothers and sisters (b x s or full-sib mating) for 20 or more consecutive generations. By this method most rapid and a maximum increase in the *coefficient of inbreeding* can be achieved. Even then it is not completely inbred in the true sense, because it gives an *inbreeding coefficient* of only 98.6 per cent, which means that on an average 1.4 per cent of the originally heterozygous genes are still heterozygous. Parent and off-spring mating may be substituted for b x s mating provided in the case of consecutive parent x offspring mating, the mating in each case is to younger of the two parents. The laboratory animals most commonly used in their inbred states are mice and rats. The chief drawbacks of inbred strains are that the animals become generally less healthy and more susceptible to diseases, and their reproductive capacity is reduced (*inbreeding depression*). In experiments on inbred strains the environment needs to be more closely controlled than in tests using hybrids or random-bred animals (Falconer, 1976).

*F<sub>1</sub> hybrids.* The first generation (F<sub>1</sub>) of a cross between two different inbred strains consists of animals that are genetically uniform but are not inbred. The F<sub>1</sub> animals, unlike inbred strains, do not suffer from *inbreeding depression* and they are improved in viability. Hence, they are less costly to produce than the inbred strains. They also exhibit increased resistance to diseases.

*Random breeding.* It means that the young animals for breeding are chosen

\* For detail guidelines refer to Special Article in Ind. J. Pharmacol. 35, 257, 2003.

without regard to their parentage and mated together without regard to their relationships; the breeding is thus random with respect to pedigree.

*Selective breeding.* It consists of choosing the individuals to be parents according to the characteristic that it is desired to change. If, for example, it is desired to increase the body weight, then the heaviest individuals are chosen to be the parents of the next generation; or if it is desired to increase the response to a certain drug, then the individuals showing the greatest response to it are chosen as parents.

### Breeding Methods

*Hand mating.* The male and the female are brought together for a brief period and then separated once the mating is over. Rabbit and hamster are mated by this method

*Pair mating.* In the case of mice, one male is mated with one female, and left together for the rest of their breeding life. In the case of rats, pregnant females should be isolated prior to delivery.

*Trio mating.* One male is mated with two females.

*Harem mating.* Up to four females are regularly mated with one male. The female is isolated soon after the pregnancy is established, and replaced by fresh females. Guinea pig is mated by this method.

### FACTORS AFFECTING THE NATURE AND DEGREE OF PHARMACOLOGICAL RESPONSES

*Species.* In rabbits intracerebroventricular injection of 5-hydroxytryptamine induces a lowering of temperature, but in cats it induces fever. Ethacrynic acid is almost inactive diuretic in the rat except in extremely high doses, but quite active in the dog. Following the same dose of hexobarbitone per unit body weight, the average sleeping time of rats is about seven times that of mice, and in dog its effect lasts for hours. Guinea pigs and humans are some 500 times more sensitive to histamine than are rats and mice. Histamine powerfully contracts the uterus of guinea pig, while relaxes that of rat. In rodents, it produces strong arteriolar constriction, in cat slight constriction, while in the dog, monkey and man arteriolar dilatation. The rat heart is known to be very resistant to cardiac glycosides. Alloxan produces diabetes mellitus in a number of species but not in guinea pig. Rabbits generally show symptoms following insulin when the blood sugar level is 45 mg percent, while dogs show symptoms only when the level is about 18 mg per cent.

*Strains.* Due to the occurrence of the hydrolyzing enzyme (atropinesterase) in the serum and liver of some genetically determined rabbits, certain strains of rabbits are exceedingly resistant to atropine. Sensitivity variations have also been noted between different strains of mice to the action of histamine, insulin and barbiturates.

*Sex.* Renal lesions following exposure to chloroform have been observed exclusively in male mice but not in female mice. Female rats are more sensitive to

certain barbiturates than are males. The male rat has a faster growth rate than the female; therefore, indiscriminate use of both males and females in a rat growth test should be avoided.

*Age.* In general, neonates tend to be more sensitive than are adults to certain drugs. For example, morphine is about four times more potent in 14-day old rats than in rats that are 28-day old. Similarly, the LD 50 of newborn rats to pentobarbitone is about one-third that of adult rats.

*Circadian or biological rhythm.* When amphetamine is given to rats at one phase of circadian system, 77.6 per cent of them survive, whereas at another phase only 6.6 per cent survive. The duration of sleep following pentobarbitone averages 104 minutes when administered at one phase, while it averages only 43 minutes when administered at another phase. The anti-cancer drug cytosine arabinoside is far more toxic at one phase of the mouse circadian system than it is at the other (Scheving, 1976).

*Isolation or group housing.* Doses of pentobarbitone or phenobarbitone, which produce full hypnosis in isolated mice, produce marked stimulation when the animals are grouped. The cardiovascular system becomes more responsive to isoprenaline in solitary-housed (6 to 8 weeks) rats compared to group-housed rats (Speiser and Weinstock, 1974).

*Season.* The antidiuretic action of vasopressin in rats was found to be greater in spring and summer, and the variability was least in winter. Mice are more resistant to insulin-induced convulsions in summer, and the slope of the regression line is steeper in winter than in summer. Frogs have been found to be less susceptible to the cardiotoxic effects of ouabain, strophanthin and digitalis during the period of July-October than during the rest of the year. This is due to the fact that they hibernate, and their metabolic rates are accelerated in the summer months. The stimulation of the vagus nerve in frogs produces predominantly inhibitory response in winter and acceleration in summer. In rabbits, a low incidence of gonadotrophin-induced ovulation occurs during the months of August-October. After oestrogen treatment, no ovulation occurs in summer and autumn, whilst large numbers ovulate in winter and spring. The rabbit uterus is more sensitive to oestrogen in spring than in autumn or early winter.

### THE RAT (*Rattus norvegicus*)

Albino rat is one of the commonest laboratory animals suitable for experimental work because of its small size and greater sensitivity to most drugs. It is also the most standardized of all laboratory animals. It can be bred to obtain pure and uniform strains, and is found to be very sturdy to withstand long periods of experimentation under anaesthesia.

There are two original strains of albino rats, *Wistar* and *Sprague-Dawley* that have been widely used throughout the world (Lane-Petter, 1976).

*Wistar rat* is a quiet, moderately prolific strain rather resistant to infection, and

has a low incidence of spontaneous tumours. The head is wide, especially in the male, and the ears are long. The tail-length is always less than the body-length.

*Sprague-Dawley rat* is more rapidly growing and more prolific than *Wistar rat*, and has a longer and narrower head, and a longer tail, which may equal the body-length. It is also less resistant to infection, especially to respiratory disease.

There are certain anatomical peculiarities that should be noted. Rodents do not vomit because they lack vomiting centre. Rats do not have any tonsil or a gall bladder. They have extremely diffuse pancreas, thus pancreatectomy is difficult to perform in order to produce a diabetic rat. The stomach shows an obvious division into two parts by a prominent white somewhat curved transverse ridge (limiting ridge). The upper two-fifth nonsecretory portion (rumen) is translucent and thinner than the lower three-fifth glandular secretory portion. Histologically also the two areas show distinct differences. The glandular portion is composed of two clearly defined areas that can be distinguished both by naked eyes as well as under a microscope. The antrum is a roughly semicircular area straddling the lesser curvature below the limiting ridge. It is relatively thin and translucent, and has a slightly irregular inner lining. The remainder of the lower two-third comprises the body of the stomach, which straddles the entire greater curvature below the ridge except for a small area above the pylorus. This is the thick portion of the stomach with mucosa thrown into folds. Shay and co-workers (1945) believe that this part of the stomach is analogous to the body of the stomach in man rather than to the fundus both anatomically and functionally. The rat being omnivorous resembles man nutritionally.

**Oestrus cycle.** The cycle makes its appearance at puberty at the age of two to three months, and the whole cycle lasts for about four to five days being divided into four stages according to the cell types found in the vaginal smear. These are as follows (Hafez, 1970) :

- i) *Oestrus* (9 to 15 h) is characterized by sexual receptivity when the female will allow copulation. During this period there are increased running activity, quivering of the ears, and lordosis in the presence of another rat. The vaginal smear shows 100 per cent cornified epithelial cells.
- ii) *Met-oestrus* (about 20 h) follows oestrus and occurs shortly after ovulation. The vaginal smear is characterized by many leucocytes with a few cornified cells.
- iii) *Di-oestrus* (60 to 70 h) is the longest of the phases, and the vaginal smear consists mainly of leucocytes.
- iv) *Pro-oestrus* (about 12 h) that follows di-oestrus is preparatory to the next oestrus phase. The vaginal smear is characterized by nucleated epithelial cells either singly or in sheets.

Vaginal smears may be taken in the following way to confirm the actual stage. Cotton swab made with toothpick is moistened with saline and gently inserted and slightly rotated within the vagina. The swab is then pressed in a drop of saline

on a microscope slide and examined under low power. An alternative and better method is to take the vaginal wash with a few drops of saline with the help of a capillary pipette fitted with a rubber teat.

### Rat as an Experimental Animal

Rats are particularly suitable for testing of psychopharmacological agents because they can be trained properly for various types of work performances including development of conditioned reflexes. They are also utilized for the assay of different hormones and for the study of oestrus cycle, mating behaviour and lactation.

These animals are also employed for the study of drugs on the blood pressure both in acute as well as in chronic or surviving experiments.

Shay and co-workers (1945) produced for the first time an acute ulcerative lesion in the forestomach (rumen) by pyloric ligation. The effects were so uniform that the Shay rat method was subsequently adopted by a number of workers for evaluation of antiulcer drugs.

The status of the rat as an experimental animal in the study of gastric secretion is unique, since a *continuous secretion* though of a low order appears in the fasting animals in contradistinction to large animals like cat, dog, etc. The mechanism of this low spontaneous secretion is not clear. Since there is some evidence that bile in the intestine is a fairly strong gastric secretagogue, the absence of gall bladder in the rat resulting in a continuous entrance of bile into the intestine may serve as a partial explanation.

For the study of gastric secretion, animals are always fasted (water allowed) for a period varying from 24 to 72 hours. Special care should be taken so that they do not get access to their stools, because they are in the habit of eating their own stools (*coprophagy*).

A quantitative method for the assay of inhibitors of acid gastric secretion has been developed (Ghosh, 1958) based on the continuous recording of acid secretion in the rat (Ghosh and Schild, 1958).

Rats are also employed for the study of analgesic drugs by the method of radiant heat application on the tail. They have been employed routinely in the toxicity studies, both acute and chronic, especially the latter, because the drug can be administered and effects observed throughout their short life span of about three years.

The 24-hour old rat is said to be physiologically similar to a 6-month old infant, and is used for evaluation of compounds supposed to be administered in infants of this age group.

It is particularly suitable for the study of the physiology of the liver, since following partial hepatectomy (60 to 70 per cent liver tissue removed) the organ regenerates almost completely in course of a week.

Rats are suitable for the testing of drugs for teratogenicity and carcinogenicity. Various isolated tissues such as uterus, stomach, colon, etc. are also routinely

## Common Laboratory Animals

employed for the study of drug actions. The uterus of the rat is inhibited by adrenaline, which forms a basis of one of the bioassay methods of adrenaline.

### THE MOUSE (*Mus musculus*)

Albino mice are the smallest laboratory animals that can be bred uniformly, are cheap and easy to handle, and being very small are sensitive to small doses of a substance. Swiss albino mouse is the most widely used strain for laboratory investigations.

#### Mouse as an Experimental Animal

Mice are employed widely in acute toxicity studies.

They are also used for the assay of insulin and analgesics, the latter specially against chemically induced pain. General screening of chemotherapeutic agents is undertaken in this species.

Specially bred mice are used for the study of problems in genetics and in cancer research.

Mice are most frequently used for the testing of drugs for teratogenicity.

The development of *nude mouse*, a hairless genetic mutant that lacks a thymus, and thus deficient in T lymphocytes, has been of fundamental interest in the field of tissue immunity and transplantation research. They are less susceptible to cancer, the reason being that they possess normal numbers of natural killer cells that kill both tumour cells and those infected by viruses (Marx, 1980). *Biege mice*, on the other hand, which lack killer cells are unusually susceptible to cancer.

Being very small and delicate the isolated tissues of mice are rarely used excepting vas deferens and ileum.

### THE GUINEA PIG (*Cavia porcellus*)

The name guinea pig has become almost synonymous with experimental animal, although in fact mice and rats are used in far greater numbers. Guinea pig or cavy has proved to be the most useful laboratory animal. Like other laboratory rodents, it is easy to keep them in captivity. They differ from other laboratory rodents, being docile in nature, and very susceptible to tuberculosis and to anaphylactic shock. They are also highly sensitive to histamine. Following histamine or anaphylactic reaction, there is bronchoconstriction, and the animal usually dies from asphyxia. Penicillin is 100 to 1000 times more toxic to the guinea pig than to the mouse. In this species, dopamine causes a fall in blood pressure. The uterus is inhibited by adrenaline. Guinea pig serum contains an enzyme asparaginase that has been shown to have some anti-leukaemic action.

#### Guinea pig as an Experimental Animal

Guinea pigs are employed for the evaluation of bronchodilator compounds against experimentally induced asthma (histamine or acetylcholine aerosols).



They are widely used in immunology, particularly in the studies of delayed hypersensitivity. Sensitization studies are best conducted in guinea pigs employing different antigens like egg albumin, horse serum, etc.

They are employed in the study of local anaesthetics as well as for the bioassay of digitalis.

Guinea pigs having sensitive cochlea are suitable for hearing experiments.

Being docile in nature and being more resistant to hypoxia than rats and mice, they are suitable for experiments on oxygen consumption.

Being the only common laboratory animal which resembles man in that it requires exogenous vitamin C, guinea pig is used in the study of ascorbic acid metabolism.

It is a suitable host for mycobacterial infection, and is used in the study of tuberculosis.

Guinea pig seems to be closer to humans than rats for the study of isoniazid toxicity.

Various isolated tissues such as ileum, tracheal chain, vas deferens, etc. are utilized for pharmacological studies of different compounds. The terminal ileum is the most common and standard preparation for preliminary screening of spasmodic or antispasmodic compounds, and is particularly sensitive to and suitable for the detection and assay of histamine and related compounds.

### THE RABBIT (*Oryctolagus cuniculus*)

Rabbits are very docile animals employed for a variety of studies. New Zealand white rabbits are widely used for the purpose. Genetically, the most interesting finding is the linkage between the gene for atropinesterase and that influences the colour of the fur (extension of black pigment in the coat). The occurrence of atropinesterase greatly shortens the pharmacologic response to atropine and this can be detected by intravenous injection of 0.1 mg/kg atropine sulphate. In atropinesterase-containing rabbits the pupillary light reflex is either not abolished or it recovers within 10 to 15 minutes, whilst in atropinesterase-free animals it is abolished (Ambache *et al*, 1964). The rabbit has got a huge caecum. The afferent *cardio-aortic nerve*, which in most species including man ascends in the trunk of the vagus, forms a separate nerve bundle called the *depressor nerve* in the rabbit. The rabbit does not appear to possess adrenergic vasodilator nerves; hence no vasomotor reversal phenomenon is demonstrable in this species. Dopamine causes fall in blood pressure in this species. Histamine tends to raise the blood pressure and produce constriction of the pulmonary vessels. Ovulation in the rabbit is related to the release of luteinizing hormone and occurs about 10 hours after coitus. Such coitus-induced ovulation is inhibited by progesterone. Uterus of the rabbit is stimulated by adrenaline.

## Common Laboratory Animals

### Rabbit as an Experimental Animal

Rabbits are employed for the pyrogen testing for intravenous fluids.

Agents affecting capillary permeability are studied by intracutaneous injection of these compounds followed by intravenous injection of some dyes like Evans blue.

Insulin and other antidiabetic drugs, curare and sex hormones are tested in the rabbit.

Its skin is relatively sensitive to irritants, so a great deal of experimentation on topical agents is conducted in rabbit.

The convenience of injecting into and withdrawing blood from the ear veins is unique in this laboratory species.

Rabbit is routinely used in serological work and for screening embryotoxic agents and teratogens.

It is especially suitable for research on reproduction as ovulation is nonspontaneous, there is no seasonal anoestrus, gestation is short, and semen is easily collected.

Isolated heart, duodenum and ileum are some of the preparations routinely employed for the testing of drugs.

### THE HAMSTER\*

Two species of hamsters are commonly used as laboratory animals, the Syrian or Golden hamster (*Mesocricetus auratus*) and the Chinese hamster (*Cricetulus griseus*).

Hamsters have chunky body with short legs, a diminutive fluffy tail and a large amount of loose skin covered with dense short soft fur. There are four toes on the front foot and five on the back, and the footpads are hairless. They have prominent cheek pouches extending up to the shoulder region. The ears are quite prominent, rounded, usually pigmented and scarcely covered with hair.

### Hamster as an Experimental Animal

Golden hamsters are used for investigation in a number of fields such as virology, cancer and nutrition research, genetics, pharmacology, and toxicology and reproductive physiology.

Chinese hamsters are most commonly used in research on diabetes, because of the high incidence of spontaneous diabetes mellitus in this species, presumably due to the deficiency of  $\beta$ -cells, or presence of defective  $\beta$ -cells in the pancreas.

This species is also used as a host in certain parasitological investigations.

Chinese hamsters have a low chromosome number (22) compared to other laboratory animals and this makes them excellent tools for cytological investigations, genetics, tissue culture and radiation research.

\* LAIS, 1981

The presence of cheek pouch in hamsters makes it a very useful animal model for research in the field of immunology. The pouch can be pulled out under anaesthesia and can be stretched over an illuminated surface for observation. The pouch in the everted position can be used as a site for the implantation of auto, homo, or hetero-grafts. The cheek pouch of golden hamster is capable of accepting many tumour grafts from other species, and their take is nearly 100 per cent.

Strips cut from the pouch of the golden hamster stomach have been used as useful *in vitro* test preparation for the assay of prostaglandins E & F (Ubatuba, 1973).

### THE CAT

Cats are common among the carnivores that are relatively easy to obtain and to use for the experimental purpose. They are used in studies on the physiology of the circulatory and neuromuscular systems because the behaviour of these systems corresponds more closely to that of man. The cat has a highly developed nictitating membrane (third eyelid), which is contracted by stimulation of the cervical sympathetic trunk, and also by a number of drugs like adrenaline, histamine, etc. In cat, morphine produces excitation of the central nervous system. Histamine produces constriction of the pulmonary vessels and relaxation of tracheal muscles. The depressor nerve in this species may run with the vagus on the right side, but separately on the left. Cats are used for the studies where stable blood pressure is required. Their vein walls are much stronger, thus easier to manipulate than those of the rabbit, the other common laboratory animal of comparable size. The uterus of the pregnant cat is stimulated, and that of virgin cat is inhibited by adrenaline.

#### Cat as an Experimental Animal

Cats are employed in acute experiments for the study of drugs affecting blood pressure.

Both anaesthetized and spinal preparations are used, the latter being particularly suitable for the assay of catecholamines.

Contractions of nictitating membrane are recorded for the investigation of ganglionic blocking action of drugs.

Cats are essential in the study of the nerve centres in the brain.

Because of their ability to produce methaemoglobinemia, cats have been found to be most suitable for the toxicity studies of compounds like acetanilide.

### THE DOG

Dogs (Mongrel, Beagles, etc.) are useful among the large laboratory animals, because they can be tamed as well as trained without much difficulty. Because of a small stomach and short intestinal tract resembling those of man, and that a dog can be conditioned to carrying a stomach cannula, it is the animal of choice for

## Common Laboratory Animals

studies on gastric secretion and digestion. Classical work of Pavlov on conditioned reflexes in the dog associating hunger and various signals is well known. In this species the cervical sympathetic and vagal nerves run together inseparably in the same trunk (vagosympathetic nerve). As a result the stimulation of nictitating membrane through the preganglionic sympathetic trunk is complicated by central vagal stimulation causing reflex variations in blood pressure.

### Dog as an Experimental Animal

Anaesthetized dogs are routinely used in the study of drugs on blood pressure in acute experiments. The size and accessibility of its vascular system coupled with its ability to learn, makes the dog invaluable for experiments on circulation that need unanaesthetized animals.

A discrete pancreas allowing ready resection enabled the classical work on diabetes to be done on the dog. Since dog develops spontaneous diabetes mellitus resembling man, hypoglycaemic drugs can be studied in these animals with advantage.

Chronically prepared gastric fistula and pouches by earlier operations are also employed for the study of gastric secretion in the dog.

### THE MONKEY

Monkeys and apes belong to the primates, the highest order of mammals that includes man. Both structurally and functionally monkeys and apes closely resemble man. For instance, they are unique among all subhuman mammals in possessing a human type of uterus, and in exhibiting regular menstrual periods. The structure of their brain also resembles that of man very closely. The high neurological development of monkeys and anthropoid apes makes them particularly suitable for studies of psychopharmacological agents. Metabolism in rhesus monkey nearly approximates that of man. Its sensitivity to drugs is also close to that of man.

### Monkey as an Experimental Animal

Primates are used in the fields of virology, parasitology, immunology and immunosuppression, nutrition, reproduction, etc.

### THE FROG AND THE TOAD

Since all amphibia absorb moisture through their skin, they must always be provided with water. It is almost impossible to breed frogs and toads in the laboratory without inducement by some hormone. The vagus nerve in the frog is a mixed one, containing the sympathetic accelerator component, the *accelerans*. The response to its stimulation varies from frog to frog and also from season to season. Sometimes an inhibition is produced and sometimes an acceleration; the former predominates in winter, while the latter in summer. Unlike in mammals where

noradrenaline serves as the neurotransmitter, in the frog and toad adrenaline is the transmitter in the sympathetic system. Hence, the frog heart falls into the range of the predominant  $\beta_2$ -adrenergic organs, and it is more sensitive to adrenaline.

### The Frog and the Toad as Experimental Animals

They are commonly used in the study of the action of drugs on the central nervous system, on the heart, on the neuromuscular junction, as well as for the diagnosis of pregnancy. For the latter, the "male frog test" is much more rapid than the older tests and equally accurate as well as simple.

Frogs have been used for determining the retinal toxicity of drugs. Light bleaches rhodopsin in frog retina in one hour and the same is resynthesized in one hour in the dark. Certain drugs can be assayed for their toxicity, since they prevent the resynthesis of rhodopsin in proportion to the dose given (Goodwin *et al*, 1957).

The biological and physiological data of common laboratory animals are presented in Tables 1.1 and 1.2.

Table 1.1  
Biological data of common laboratory animals\*

Parameter	Rabbit	Guinea pig	Rat	Mouse	Hamster	
					Golden or Syrian	Chinese
Typical adult weight (g)	1500-5000	800	250	20-40	80-90	35-40
Average life span (years)	4-6	3-5	2-3	1.5-2.5	2-3	2-3
Average age suitable for experiment (months)	6	3	1.5	0.75	1	1
Age of first mating (months)	6-7	3	2.5-3	1.5-2	2-3	2-3
Breeding habits (1 male to number of females)	1	6	5	3	1	1
Gestation period (days)	31	68	21-23	19-21	16	21
Average litter size	6-8	3-4	8-10	6-12	5-7	4-5
Age at weaning (days)	45	14-21	21	21	20-25	21
Weight at weaning (g)	600-1500	160-230	35-45	10-14	30-40	6-8
Number of litters per year	4	4	6	8-10	-	7
Duration of sexual life (months)	36	24	12	12	-	12
Rest period of females between mating (days)	20	15	15	15	-	15

\* Based on LAIS Centre News (1980-82)

- Data not available

Table 1.2  
Some important physiological data of laboratory animals\*

Parameter	Rabbit	Guinea pig	Rat	Mouse	Hamster Golden
Daily food intake (g)	150-300	40-50	10-20	4-5	10-14
Heart rate (beats/min)	130-300	260-400	300-500	330-780	318-412
Respiratory rate per min	38-60	70-104	65-180	84-230	33-127
Tidal volume (ml) (air inspired/breath)	19-24	1.8	1.5	0.1-0.23	0.83
Arterial B.P. systolic	110	77	116	113	108
diastolic	80	47	90	81	77
Hb g/100 ml	8-15	8-15	12-17.5	10-19	16.8
R. B. C. (million/cmm)	4.5-7.0	5-6	7.2-9.6	4.9-12.5	7.5
W. B. C. (thousand/cmm)	6-13	4-11	6-12	4-12	7.62
Blood volume (% B.W.)	4-8	6-12	6-7	7-9	6-9

\* Based on LAIS Centre News (1980-82)

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# Some Standard Techniques

## Bleeding and Intravenous Injection

*Mouse and rat.* Both collection of blood as well as injection can be carried out by tail venipuncture in these species. In restrained (unanaesthetized) animals lateral or dorsal veins are dilated by dipping the tail into water at 40 to 50°C or by rubbing with xylol and then cleaning the part with some disinfectant. The tail is grasped between the thumb and the index finger, and the needle (25 to 27-gauge and ½ to 1" long fitted with 1 or 2 ml syringe) is introduced near the distal portion of the tail with the bevel up, and the injection made with a uniform speed. For bleeding, a gentle aspiration is applied to avoid collapse of the wall of the vein obliterating the needle opening. Another way of collecting blood repeatedly is by cutting the tip of the tail with some sharp instrument. Blood can also be collected by heart puncture in anaesthetized animals, or sometimes by decapitation. In the latter procedure, the head of the anaesthetized animal is quickly severed at the neck with a pair of heavy shears and the body is held over a container. The most efficient method of collecting blood in rats and mice causing least stress to the animals is from the orbital sinus with the help of a capillary tube. The animal is restrained in such a way that the loose skin of the head is tightened while holding the head with the left hand. With the help of the index finger the eye is made to protrude by traction on the skin adjacent to the eye. The thumb is pressed just behind the angle of the jaw resulting in the engorgement of the retro-orbital plexus. The tip of a Pasteur pipette is then inserted at the medial canthus into the retro-orbital plexus with gentle rotation by the other hand. As the vessels are ruptured blood wells up in the periorbital space. The tip of the pipette is then slightly withdrawn so that the blood flows into the pipette by capillary action. Bleeding stops once the normal ocular pressure is allowed to impinge on the venous plexus by releasing the animal (Krans, 1980).

*Guinea pig.* Small samples of blood may be obtained by simple venesection of the marginal ear vein. Larger quantities of blood may be obtained either by heart puncture under anaesthesia or by severing the carotid artery after stunning or inducing anaesthesia. In males, intravenous injection can be made through the penile veins seen along either side of the extruded penis in restrained or anaesthetized animal.

*Rabbit.* The lateral margin of the ear is shaved and swabbed with a disinfectant. The ear is grasped between the thumb and the index finger in a well-restrained animal. Using a 20-gauge needle with the bevel up, venipuncture is made at a site immediately proximal to the thumb along the marginal vein (which is relatively

immobile). Very gentle aspiration must be applied in order to avoid collapse of the vein. Injection can also be made through the same vein. Another method is to make a cut in the marginal vein or the central artery of the ear near its tip. The vein is occluded near the base, the artery is gently tapped near the animal's head and blood collected in a container. A large quantity of blood may be obtained easily by this method. The bleeding is stopped by pressing a little cotton wool held in position with bulldog clamp.

*Dog and cat.* Cephalic vein on the dorsal surface of the foreleg over the radius is usually used for injection or bleeding.

### Volume to be Injected

The volume to be administered should be large enough to measure accurately, and small enough to avoid undue rapid change in the blood volume. The usual intravenous volume for small animals is between 1 and 10 ml/kg body weight, the maximal volume for mouse and rabbit being 0.4 ml and 10 ml respectively. The volume of intramuscular injection is more limited, being about 0.25 ml for mouse, 0.5 ml for rat, 1 ml for rabbit and 2 ml for dog in order to allow reliable absorption from the injection site.

The following formula can be used for the calculation of volume, strength or amount of a drug to be injected:

$$V = (BW \times A) / S \qquad A = (V \times S) / BW \qquad S = (BW \times A) / V$$

where V = volume of the drug solution, A = amount of drug in mg/kg, S = strength in mg/ml of the drug solution, and BW = body weight of the animal in kg.

For example, the volume of a 25 per cent urethane to be injected to a 180 g rat on the basis of 175 mg per 100 g body weight is obtained thus:

$$V = (0.18 \times 1750) / 250 = 1.26 \text{ ml}$$

### VENTILATION RATE

The ventilation rate for dog, cat and rabbit is 20 strokes per minute, each of 10 to 15 ml/kg (200 to 300 ml/kg/min), and for rat 50 to 70 strokes per minute, each of 8 to 10 ml/kg (400 to 700 ml/kg/min).

### INTRAGASTRIC ADMINISTRATION

*Mouse.* A polythene tubing 2 to 3 cm long sleeved on an 18 to 20-gauge blunted hypodermic needle, or a Eustacean catheter may be used for the purpose. The animal is grasped securely by the nape of the neck holding the whole animal with the left hand. The polythene tubing is introduced laterally through the intradental space, and with rotating motions gently advanced into the oesophagus. When it is relatively certain that the tube is at the desired level, the fluid is introduced slowly.



A solution or a suspension in 5% gum acacia is administered in a volume of 0.5 ml per 20 g body weight.

*Rat and guinea pig.* A 15 to 16-gauge hypodermic needle of 7.5 to 10 cm length is blunted and a small ball of solder applied around the tip, and a gentle bend (20 to 30°) is made about 2 cm proximal to the solder. The technique is almost similar as in the case of mouse.

*Rabbit.* An assistant sitting on a chair with the rabbit on his knees holds the animal. With pressure to the cheeks at the corners of the mouth the animal opens its mouth, when a wooden gag with a small hole in the centre is placed in the mouth behind the incisors. A No.12 French catheter (4 mm dia) or a No.9 soft rubber catheter (5 mm dia) moistened with glycerine is inserted through the central hole with right hand as the left hand controls the gag. The tube passes over the base of the tongue swallowed slowly. The outer end of the tube can be placed under water or near the operator's ear to check for accidental tracheal intubation. The outer end of the tube should be occluded as it is removed.

### PROCEDURES FOR RENDERING ANIMALS UNCONSCIOUS

There are various methods by which an animal is rendered unconscious so that it does not feel pain, and also killed painlessly at the end of the experiment.

*Mice, rats, guinea pigs and rabbits.* The commonest, quickest and the most humane method of killing these animals is by preliminary *stunning* (crushing the skull) followed by cutting the throat for bleeding. *Stunning* is carried out either by a smart blow behind the head or on the back of the neck with a blunt instrument, or by striking the back of the head and neck very hard against a sink or a table edge. The immediate effect of stunning is a stiffening of all the muscles followed by a series of convulsions followed by gradual relaxation of the limbs and body. The convulsions result from massive discharges from the central nervous system resulting from trauma to the crushed brain. There are no indication of suffering on the part of the animal. If there is any doubt about the animal regaining consciousness, the head should be cut off immediately.

*Frogs.* They can be rendered insensitive to pain simply by chilling them to about 4°C. The simplest way to do this is to place them in an ordinary refrigerator until the desired state of immobility is reached, or they can also be immersed in ice water. They are also rendered unconscious by *pithing*, i.e. by destroying the central nervous system. *Pithing* is done by grasping the body of the frog firmly in one hand and depressing the head with the thumb so that the connection between the skull and the vertebral column is opened. A sharp-pointed probe of suitable length is inserted through the skin in between the skull and the atlas, and pushed into the cranial cavity destroying the brain completely. If the spinal cord is also to be destroyed, the probe is removed and reinserted into the open end of the spinal canal and pushed downward as far as possible. This will sever the central connections of all spinal nerves leaving the animal flaccid. A *singly pithed frog* is

## Some Standard Techniques

one in which only the brain is destroyed. In a *doubly pithed* frog the spinal cord as well as the brain are destroyed. Unless otherwise indicated the *double pithing* procedure is always employed.

### CHEMICAL EUTHANASIA

It is the painless death produced by administration of chemical poisons. Intravenous or intracardiac magnesium sulphate injection is an excellent lethal agent from the point of view of availability, cost and safety. A saturated solution (or about 25 per cent if in dehydrated form) is injected in a dose of 15 g/kg body weight. If an inhalation agent is preferred, chloroform can be used with advantage. A large volume of air may be injected intravenously when nothing else is available.

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# Anaesthetics Used in Laboratory Animals

## CHLORALOSE

It is a compound of chloral and glucose prepared by heating equal parts of anhydrous glucose and chloral, when both  $\alpha$ -chloralose (active form) and  $\beta$ -chloralose (inactive form) are formed. Chloralose is the active form ( $\alpha$ -chloralose) freely soluble in hot water, alcohol and ether, and slightly so in cold water. It is prepared as one per cent solution by boiling in 0.9% NaCl (saline) or in distilled water, and administered intravenously or intraperitoneally at a temperature of 30 to 40°C before the chloralose comes out of solution.

It is suitable only for acute (nonrecovery) experiments, usually in dogs and cats, inducing surgical anaesthesia for three to four hours or longer. It has the advantage of greater constancy of the depth of anaesthesia. The respiration and circulation are not depressed, and the blood pressure is well maintained usually on the higher side. Reflexes are not depressed but may be slightly exaggerated including responses to bilateral carotid occlusion. Pressor response to catecholamines is least reduced, if at all. In the rat, the pressor response to noradrenaline and reflex bradycardia are potentiated (Brezenoff, 1973). There may be paddling movements of legs, jerking of the body or nodding of the head; but there is good relaxation of muscles, and cutting of tissues does not evoke reflex movement. Chief disadvantage is its low water solubility, but can make up 10 per cent solution in polyethylene glycol. Autonomic reflexes are disturbed in an unpredictable manner (Bass and Buckley, 1966). It is not a suitable anaesthetic for rabbits since animals are narcotized rather than anaesthetized, large volume needed, and may produce convulsion on slight stimulation.

## URETHANE (Ethyl carbamate)

It is readily soluble in water giving a neutral solution. Usually 25 per cent solution in water is used.

It is suitable only for acute experiments since it has delayed toxic effect on liver, and may also cause agranulocytosis and pulmonary adenomata; has little or no effect on nerve transmission and produces little reflex depression. Basal blood pressure is uniformly lower than with chloralose in dogs (Kolatkar *et al.* 1973). Pressor response to catecholamines is most reduced compared to other anaesthetics. Especially suitable for rabbits and rats, duration of anaesthesia being three to four

hours or more. Mice develop an exceptionally high incidence of lung tumours regardless of the route of administration. Frogs can be anaesthetized by placing them in a covered beaker containing 5 to 10 per cent urethane solution.

### BARBITURATES

Barbiturates interfere with nerve impulse transmission both in the central nervous system and in the ganglia producing depression of cardiovascular and spinal cord reflexes. Marked slowing of respiration is produced following barbiturates. In rabbits pedal reflex (leg retraction) is lost first, then pupillary and finally palpebral reflex (*cf.* ether with which the order is reversed).

Phenobarbitone sodium and barbitone sodium are used for prolonged experiments.

Pentobarbitone sodium (Nembutal) 6% solution (dissolved in 10% alcohol) is used for rapid anaesthesia, and small doses repeated at intervals for maintenance of anaesthesia. Since a standard dose of pentobarbitone can produce varied depths of anaesthesia, this difference in responsiveness could cause considerable variability between animals, unless depth of anaesthesia is carefully monitored (Brezenoff, 1973). Because of strong vagolytic effect, pentobarbitone may not be an ideal anaesthetic for evaluation of cardiovascular drugs (Olmsted and Page, 1966).

Thiopentone sodium (Pentothal) is used for surgical operations of short duration. It produces rapid induction with minimum excitation.

### PARALDEHYDE

It has a wide margin of safety because it depresses only the cerebrum and not the medullary centres. Intravenous injection is likely to produce cardiac dilatation and pulmonary congestion and oedema. Under its influence the basal blood pressure as well as the response to vasopressor and depressor drugs are low. Bilateral carotid occlusion produces poor pressor response or even a depressor response (Kolatkar *et al.*, 1973).

### MAGNESIUM SULPHATE

A 20% magnesium sulphate solution 5 ml/kg intravenously produces anaesthesia for about an hour; calcium gluconate intravenously will counteract its depressant effect immediately. Its principal use is in producing euthanasia.

Responses to different anaesthetics in dog are presented in Table 3.1. Changes in cardiac and respiratory rates following drugs and nerve stimulation in anaesthetized dogs are shown in Table 3.2. Doses of different anaesthetics in laboratory animals are presented in Table 3.3.

Table 3.1  
Responses to different anaesthetics in dog

Parameters	Chloralose	Pentobarbitone sodium	Phenobarbitone sodium	Paraldehyde
Dose per kg and route	83.5 mg i.v. (80-100)	35.0 mg i.p.	191.2 mg i.p. (180-200)	2.13 ml i.m. (2-2.5)
Loss of consciousness (min)	10.0 (9-11)	11.7 (10-15)	51.5 (40-60)	73.7 (42-95)
Onset of anaesthesia (min)	17.9 (15-20)	16.8 (14-20)	79.3 (70-94)	97.6 (62-115)
Blood pressure (mmHg)	187.0 (170-200)	103.0 (90-120)	100.0 (90-110)	102.8 (90-120)
Heart rate/min				
Before anaesthesia	98.8	85.7	88.2	117.0
After anaesthesia	107.2**	107.2**	94.8	127.8*
Respiratory rate/min				
Before anaesthesia	42.2	31.6	53.6	36.0
After anaesthesia	38.8**	12.8**	13.3**	25.0**

Each value is a mean obtained in 10 animals; ranges given in parantheses

\* P < 0.05,

\*\* P < 0.01 by *t* test on paired samples

Table 3.2  
Changes in cardiac and respiratory rates following drugs, carotid occlusion and vagal stimulation in anaesthetized dog

Anaesthetic		Basal	Adrenaline	Noradrenaline	Isoprenaline	Acetylcholine	Carotid occlusion	Vagal stimulation (peripheral)
Chloralose	H.R.	107.2	122.8**	107.6	178.2**	84.2**	99.4**	84.8**
	R.R.	38.8	41.0**	45.2**	49.2**	49.8**	44.4**	45.6**
Pentobarbitone	H.R.	107.2	118.6**	110.8**	156.8**	84.0**	99.4*	86.8**
	R.R.	12.8	14.2*	13.6	13.4	22.2**	13.2	13.4
Phenobarbitone	H.R.	94.8	113.0**	94.6	159.6**	76.8**	88.4*	73.4**
	R.R.	13.3	15.8**	15.8**	15.8**	22.6**	15.0*	16.4**
Paraldehyde	H.R.	127.8	144.8**	131.2	190.2**	112.8**	128.6	107.4**
	R.R.	25.0	26.2	24.0	24.0	35.6**	23.8	27.6

Each value is a mean obtained in 10 animals

H.R. = Heart rate/min ;

R.R. = Respiratory rate/min

\* P < 0.05,

\*\* P < 0.01 by *t* test on paired samples

## Anaesthetics Used in Laboratory Animals

Table 3.3  
Doses of anaesthetics in laboratory animal

Anaesthetic	Species	Solvent & strength	Dose per kg & route	Remarks
Chloralose	Dog	1% aq. soln. (hot) 10% in polyethylene glycol	80 to 120 mg i.v. 100 mg i.v.	Vein cannulated under ether
	Cat	1% aq. soln. (hot)	80 mg i.v.	
		2% aq. or saline suspension 10% in propylene glycol	80 to 100 mg i.p. 100 mg i.p.	
	Rat	10% in propylene glycol	80 mg i.p.	
Urethane	Dog	25% aq. soln.	1.5 g i.v.	Dose is exceedingly variable, usually slowly injected to effect
	Cat	25% aq. soln.	1.0 to 1.5 g i.v.	
	Rabbit	25% aq. soln.	0.5 to 1.75 g i.v.	
		50% aq. soln.	1.5 to 2 g i.p.	
	Guinea pig	25% or 50% aq. soln.	1.5 g. i.p.	
	Rat	25% aq. soln.	1.25 to 1.75 g i.m. or s.c.	
	20% aq. soln.	1.5 g. i.p.		
Paraldehyde	Dog & Cat		1.2 ml i.p.	
			2.1 ml i.m.	
Pentobarbitone sodium* (Nembutal)	Monkey	6% soln.	25 mg i.p. or i.v.	Supplements of 5 mg/kg i.v. as needed during experiment
	Dog & Cat	6% soln.	30 to 50 mg i.v. or i.p.	
	Rabbit	6% soln.	50 to 60 mg i.v.	
	Guinea pig	1% soln.	30 to 50 mg i.p.	
	Rat & Mouse Frog	0.6% soln.	30 to 60 mg i.p. 50 mg intra-abdominally	
Phenobarbitone sodium	Dog & Cat	10% aq. soln.	180 to 200 mg i.p.	Deep anaesthesia in about 15 min and duration 1 to 2 h.
	Dog	10% aq. soln.	80 mg i.v.	

\*Dissolved in 10% alcohol

Table 3.3 (Contd.)

Anaesthetic	Species	Solvent & strength	Dose per kg & route	Remarks
Thiopentone sodium	Dog	2.5% fresh soln.	12 to 16 mg i.v. slowly to effect	For brief duration
			20 to 26 mg i.v. slowly to effect	For longer duration
<b>COMBINED ANAESTHETICS</b>				
Chloralose & Pentobarbitone sodium	Dog		Chloralose 80 mg & Pentobarbitone 10 mg i.v.	
	Cat		Chloralose 70-100mg & Pentobarbitone 2.5 to 6.0 mg i.p.	
	Guinea pig		Chloralose 100 mg & Pentobarbitone 30 mg i.p.	
	Rat & Rabbit		Chloralose 100-120 mg & Pentobarbitone 30 mg i.v.	
Chloralose & Urethane	Dog		Chloralose 50 mg & Urethane 500 mg i.v.	
	Cat		Chloralose 40-60 mg & Urethane 400-500 mg i.p	
	Rabbit		Chloralose 60-72 mg & Urethane 1.0 to 1.2 g i.v.	
Urethane & Pentobarbitone sodium	Rabbit		Urethane 700 mg & Pentobarbitone 40 mg i.p	
	Guinea pig		Urethane 1.5 g & Pentobarbitone 6 mg i.p.	
	Rat		Urethane 400-500 mg & Pentobarbitone 5-20 mg i.p.	

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# Some Standard Drug and Salt Solutions and Some Useful Information

## PHYSIOLOGIC SALT SOLUTIONS FOR ISOLATED ORGAN BATH

All solutions should be prepared with fresh glass-distilled water, and the reagents must all be of analytical grade. When a salt containing water of crystallization is used instead of an anhydrous salt, the weight of the salt should be suitably adjusted. For instance, when 0.2 g of anhydrous  $\text{CaCl}_2$  is required, a solution can be prepared from, say,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  by the following formula:

$$\begin{aligned} X &= 0.2 \text{ g} \times [(\text{mol. wt. of } \text{CaCl}_2 \cdot 2\text{H}_2\text{O}) / (\text{mol. wt. of } \text{CaCl}_2)] \\ &= 0.2 \text{ g} \times 147/111 = 0.26 \text{ g} \end{aligned}$$

where X is the amount of salt to be weighed. Similarly, when the salts are expressed in terms of molarity, the molecular weight of the salt with or without water of crystallization should be taken into account.

*Stock salt solution.* Physiologic salt solutions should be prepared fresh on the day of the experiment. It is, however, convenient to prepare a 20-fold stock concentrate of the chlorides, from which different salt solutions can be prepared. These concentrated solutions can be diluted according to the need. The required amount of sodium bicarbonate is dissolved in sufficient volume of water and added at the time of setting up the experiment, since calcium carbonate is liable to be precipitated if the calcium and the bicarbonate are kept long together, or mixed in concentrated solution, or even when heated together in dilute solutions. The presence of glucose tends to encourage bacterial growth at room temperature; hence, it is either added freshly weighed, or from a hypertonic concentrated solution kept in the refrigerator.

*pH.* The pH of the various commonly used physiologic salt solutions varies usually between 7.3 and 7.4. At lower pH the tonus of the preparation tends to decrease, thereby altering the effect of drugs.

*Temperature.* In order to get consistent effect, it is important to maintain the temperature of the bath solution at a specified level. For instance, when the bath temperature is decreased below  $37^\circ\text{C}$ , the tonus of the intestine is increased, the contractions become smaller, and the contraction and relaxation time increased.

*Aeration.* Air, oxygen or oxygen with 5%  $\text{CO}_2$  is needed for the proper



functioning of the tissues. Besides providing oxygen to the tissue, stream of gas-bubbles also stir the bath solution thereby facilitating diffusion of drugs added to the bath. The solution in the bath should be changed frequently, because prolonged aeration tends to alter the pH. In an experiment of long duration, moderate aeration should be used. In case of doubt, the pH of the bath solution should be checked.

### Composition of Some Physiologic Salt Solutions

Composition of some commonly used physiologic salt solutions is given in Table 4.1

The salt solutions contain a mixture of cations, anions and glucose in distilled water. All solutions are to be made up in double distilled deionized water, final pH of which is to be adjusted and maintained at 7.4 by the addition of NaOH. In the depolarizing solutions, KCl is substituted for an equivalent amount of NaCl to maintain isotonicity of the solution.

*Ringer, Frog-Ringer and De Jalon* solutions do not contain any Mg or  $PO_4$  ions. *Frog-Ringer* solution may also be prepared by adding 400 ml distilled water to one litre of *Ringer (Locke)* solution (single glucose).

The composition of *De Jalon* is same as that of *Ringer-Locke* except that it contains one-fourth the amount of  $CaCl_2$  and half the amount of glucose.

*McEwen* solution contains sucrose in addition to glucose.

*Calcium-free Ringer* solution is identical with *Ringer* solution except that  $CaCl_2$  is omitted.

*Calcium-free depolarizing Ringer* solution contains (mM) NaCl 94.0; KCl 60.0;  $NaHCO_3$  6.0; and dextrose 11.0.

*Zero-calcium depolarizing Ringer* solution has the same composition as *calcium-free depolarizing Ringer* solution except that it contains in addition EGTA\* 0.01.

In order to deplete the isolated tissue of calcium, Na-EDTA 0.3 mM can be added to the *Ca-free Krebs* solution (*Zero-Ca EDTA* solution).

*High calcium (50 mM)* solution is prepared by removing bicarbonate and adjusting pH to 7.4 with 5 mM Tris-HCl buffer.

*Potassium-rich Krebs* is made by increasing KCl concentration up to 47.3 mM potassium at the expense of an equivalent amount of NaCl.

*Low  $Na^+$  Krebs* (glucose 5.55 mM) solution (63 mEq/l) is prepared by reducing NaCl to 38 mM and adding sucrose 150 mM to maintain the tonicity of the solution.

*Low  $Na^+$  Krebs* solution (25 mEq/l) is prepared by omitting NaCl completely and adding sucrose 220 mM.

*$K^+$ -free Krebs* solution is prepared by omitting KCl and  $KH_2PO_4$ , and adding  $NaH_2PO_4$  1.2 mM and sucrose 9.5 mM.

*Glucose-free Krebs* solution is prepared by omitting glucose.

*Krebs* solutions may contain  $2 \times 10^{-5}$  M choline in order to feed the cholinergic nerves, and preserve their acetylcholine stores in coaxially stimulated isolated

\* Ethyleneglycol-bis-( $\beta$ -aminoethyl ether)-N,N-tetra acetic acid

Table 4.1  
Composition of some physiologic salt solutions

Composition	Frog-Ringer	Ringer or Ringer-Locke (Locke)	Modified Kreb's-Ringer's bicarbonate	Tyrode	Krebs- Henseleit (Krebs)	McEwen	Hukovic
NaCl (58.45)	110 (6.50)*	154 (9.0)	120 (7.0)	137 (8.0)	118 (6.9)	113 (6.6)	113 (6.6)
KCl (74.56)	1.9 (0.14)	5.6 (0.42)	0.8 (0.06)	2.7 (0.2)	4.7 (0.35)	5.6 (0.42)	4.6 (0.34)
CaCl <sub>2</sub> (110.99)	1.1 (0.12)	2.2 (0.24)	2.6 (0.09)	1.8 (0.2)	2.5 (0.28)	2.2 (0.24)	2.5 (0.28)
MgCl <sub>2</sub> (95.23)	-	-	-	0.1-1.0 (0.01-0.10)	-	-	-
MgSO <sub>4</sub> ·7H <sub>2</sub> O (246.39)	-	-	0.67 (0.16)	-	1.2 (0.28)†	-	1.1 (0.26)
NaHCO <sub>3</sub> (84.00)	2.4 (0.2)	6.0 (0.5)	27.5 (2.3)	11.9 (1.0)	25.0 (2.1)	25.0 (2.1)	25.0 (2.1)
NaH <sub>2</sub> PO <sub>4</sub> (119.97)	0.06 (0.008)**	-	-	0.4 (0.05)	-	1.2 (0.16)	-
KH <sub>2</sub> PO <sub>4</sub> (136.08)	-	-	1.2 (0.16)	-	1.2 (0.16)††	-	1.1 (0.15)
Glucose (180.16)	11.1 (2.0)	5.55(1.0) or 11.1 (2.0)	10 (1.8)	5.55 (1.0)	5.55 (1.0) or 11.1 (2.0)	11.1 (2.0)	11.1 (2.0)
Sucrose (342.31)	-	-	-	-	-	13.1 (4.5)	-

\*Values are in mM (g/l). Values in parentheses against salts indicate mol.wt.

†MgCl<sub>2</sub> may be replaced in its place.

††NaH<sub>2</sub>PO<sub>4</sub> may be replaced in its place.

\*\*May be omitted.

guinea pig ileum.

*Ringer* solutions are aerated with  $O_2$  or air, and used for mammalian isolated heart and other tissues; frog-Ringer is used for frog heart and tissues.

*Tyrode* solution is aerated with air,  $O_2$ , or 5%  $CO_2$  in  $O_2$  and used for mammalian smooth muscles.

Krebs, modified Krebs-Ringer's bicarbonate and McEwen solutions are aerated with 5%  $CO_2$  in  $O_2$  and used for mammalian isolated organ, specially for nerve responses.

### THE DRUG SOLUTIONS

The concentrated stock solutions of drugs may be made up in distilled water because some compounds are appreciably less soluble in physiologic salt solutions than in water. Some drugs, particularly catecholamines, are prepared in dilute hydrochloric acid or ascorbic acid solution to minimize oxidation. The final dilution should be made with isotonic saline or physiologic salt solution. Most of the drugs are freshly diluted immediately before use in 0.9% (w/v) NaCl solution (saline) containing 0.01 N HCl.

In order to prepare a drug solution accurately, a bigger amount that can be weighed accurately may be taken to prepare a stronger solution. The final solution may be prepared by dilution in stepwise fashion.

#### Drug Concentration

In isolated organ experiments, it is usual to describe the drug concentration as the weight of the drug contained in 1 ml of the bath solution. Thus, the concentration of histamine in a stock solution containing 1 mg per ml is  $10^{-3}$  g/ml. When 0.2 ml (0.2 mg) is added in organ bath of a volume of 10 ml, the final concentration in the bath solution, which is the usual way of expression, should be 20  $\mu$ g (0.02 mg) per ml or  $2.0 \times 10^{-5}$  g/ml.

*Molar concentration of drugs.* One great advantage of expressing the drug concentration in terms of molarity is that it is not necessary to specify the nature of the salt used. Thus, the comparisons between drugs and the results obtained by different workers become easier and more rational. It is the active mass of a drug rather than the actual weight that is more important in drug action. A drug is usually available in a number of salt forms, e.g. acetylcholine is available as the chloride, bromide or iodide salts. If the concentration is expressed on a weight rather than on a molarity basis, the chloride salt of acetylcholine will appear more active than the bromide or the iodide salt, because of its lower molecular weight.

A *mole* of a particular substance is the number of grams equal to the molecular weight of the substance (1 mole = 1g mol. wt.).

A *molar solution* contains one mole or gram molecular weight of the solute in one litre of solution (1M = 1 mole/l = 1 mmole/ml).

*Molarity (M)* is a number that expresses the number of moles of a substance in

one litre of solution. The molar concentration of a substance X is usually denoted by the symbol [X].

### Conversion Factors

A. To convert per cent to molarity and *vice versa*

$$\text{Percent} = (M \times \text{mol.wt.}) / 10.$$

$$M = (\text{Per cent} \times 10) / \text{mol.wt.}$$

B. To convert mg/100 ml to mEq / l and *vice versa*:

$$\text{mg/100 ml} = (\text{mEq/l} \times \text{mol.wt.}) / (10 \times \text{valence}).$$

$$\text{mEq/l} = (\text{mg/100 ml} \times 10 \times \text{valence}) / \text{mol.wt.}$$

C. To convert volume of titratable acidity in a volume of gastric contents to mEq/l

$$V_1 \times C_1 = V_2 \times C_2$$

where  $V_1, C_1$  are given volume and concentration of the titratable acid, and  $V_2$  is the volume of the gastric content.  $C_2$  is calculated in terms of normality (N), which is same as Eq/l; from this mEq/l is found out.

*Example:* 5 ml of gastric contents are used for titration, and titration requires 2.2 ml of 0.1N NaOH; find the titratable acidity ( $C_2$ ) in mEq/l.

$$V_1 \times C_1 = V_2 \times C_2$$

$$2.2 \times 0.1 = 5 \times C_2$$

$$C_2 = (2.2 \times 0.1) / 5 = 0.044 \text{ Eq/l} = 44 \text{ mEq/l.}$$

D. To convert percentage strength of a solution into mg/ml:

Shift the decimal point one place to the right e.g. 0.2 per cent solution contains 2 mg/ml.

### PREPARATION OF SOLUTIONS OF SOME UNSTABLE AND / OR INSOLUBLE COMPOUNDS

#### Catecholamines

Stock solutions of catecholamines (noradrenaline, adrenaline, isoprenaline and dopamine) 1 mg/ml are prepared in 1% ascorbic acid solution to minimize oxidation. Disodium EDTA 10 mg/ml may also be added to the stock and working solutions in order to prevent catalytic oxidation by traces of heavy metals. The drugs are freshly diluted before use in 0.9% NaCl containing 0.01 N HCl. Alternatively, an acidulated saline may be prepared by adding one or two drops of 1 N HCl per 100 ml of 0.9% NaCl.

#### Acetylcholine

Used as chloride, which is highly hygroscopic and unstable in aqueous solution; hence should be prepared fresh at the time of the experiment. It is quite stable at

pH 4 and becomes less stable at higher pH values. Solutions just acid to litmus paper are satisfactory. Stock solution of acetylcholine is made up in 5% (w/v)  $\text{NaH}_2\text{PO}_4$  solution so as to contain 1 mg/ml.

### Histamine

Used as acid phosphate (diphosphate) or dihydrochloride. Since the two salts differ greatly in their molecular weights, histamine is usually expressed in terms of free base. The molecular weight of the base and different salts, and the relative amount of free base in each salt are as follows:

<i>Histamine</i>	<i>Mol.wt.</i>	<i>Proportion of base</i>	<i>Equivalent of 1 mg base</i>
Free base	111.1	1.00	-
Diphosphate	307.1	0.36	2.75 mg
Dihydrochloride	184.1	0.60	1.70 mg

This means that to administer a given amount of histamine base we have to use approximately three times the weight of acid phosphate, or one and a half times the weight of the dihydrochloride. Stock solution of histamine should be kept at pH 4. For accurate quantitative work, it is advisable to prepare fresh solution from the powder on the day of the experiment.

### Physostigmine or Eserine

Eserine as sulphate or salicylate is used to sensitize the frog rectus muscle to acetylcholine by adding eserine sulphate 5-10  $\mu\text{g}$  per ml in Frog-Ringer solution. The preparation is sensitized 10-fold, starting about 10 minutes after eserine treatment and reaching maximum by 30 minutes. Solid and solutions turn red on exposure to heat, light or air, and on coming in contact with traces of metals. Hence, it should be kept in air-tight and light-resistant containers. Stock solution (0.1% or 1%) is made in acidulated saline and kept in cold; deterioration is shown by the appearance of a pink colour.

### Reserpine

Solution is prepared by one of the following procedures immediately before use: (i) dissolved in 20% ascorbic acid in a concentration of 10 mg/ml, (ii) dissolved in minimum amount of glacial acetic acid and diluted with distilled water to give a concentration of 1 mg/ml (final pH 5.3), (iii) dissolved in 10% ascorbic acid and pH adjusted to 4.8 with 5%  $\text{NaHCO}_3$ , or (iv) dissolved in 30% propylene glycol. Stock solution (5 mg/ml) is made by dissolving 100 mg reserpine in 2 ml glacial acetic acid, 2.5 ml propylene glycol, 2.5 ml 95% ethanol and sufficient water to make 20 ml.

### Phenoxybenzamine

It is dissolved in 95% ethanol containing 0.001 ml of 10 N HCl per ml to give a 100 mM stock solution. May also be dissolved in 4:1 mixture of propylene glycol and ethanol.

### $\alpha$ -Methylparatyrosine HCl ( $\alpha$ -MPT)

Solution (30 mg/ml) is prepared in 0.5 M phosphate buffer saline (pH 7.4) and sufficient 1N NaOH added just to effect solution and stored at 0°C.  $\alpha$ -MPT may also be suspended in water with 0.5% carboxymethylcellulose and 2.5% Tween 80.

### p-Chlorophenylalanine (PCPA)

Always to be stored at 0°C. Before use it is dissolved in 1 N NaOH, and pH adjusted to 8.9 with 1 N HCl, or suspended in 1% carboxymethylcellulose to give 30 mg/ml solution.

### 6-Hydroxydopamine HBr (6-OHDA)

Always to be stored at 0°C. Before use the salt is dissolved in cold 0.9% NaCl containing 1 or 10 mg/ml ascorbic acid. May also be dissolved in 0.001 N HCl that had been gassed with nitrogen.

### Pimozide

It is dissolved in 0.1% (w/v) tartaric acid and pH adjusted to 5.5 with 0.1N NaOH. Stock solution (10mg/ml) is prepared by dissolving 100 mg in 3 drops of glacial acetic acid and 3 drops of absolute alcohol before making up to a final volume of 10 ml with hot 5% glucose solution. Subsequent dilutions are made in saline before use.

### Haloperidol

The solution is prepared by one of the following methods: (i) dissolved in distilled water by drop-wise addition of 1 N HCl, (ii) dissolved in dilute lactic acid and pH adjusted to 4.8 to 6.0 with 0.1N NaOH, or (iii) dissolved in propylene glycol:ethanol (60:40) acidified with HCl, volume made up with saline to give a final concentration of 1 mg/ml.

### Prostaglandin E's

Stock solution is prepared by dissolving 2 mg/ml in 90% ethanol. PGE's 1 mg is dissolved in 0.1 ml 95% ethanol and 0.9 ml of 0.02% (w/v)  $\text{Na}_2\text{CO}_3$  solution before each experiment.

### Aspirin

It is used as sodium salt by mixing acetylsalicylic acid with sodium bicarbonate

in the ratio of 180:100 and dissolving in water. May also be suspended in 1% carboxymethylcellulose.

#### **Indomethacin**

It is dissolved by adding ethanol 0.4 to 0.8 ml to 10 to 20 mg powder, and then diluting to 1 mg/ml with 0.9% NaCl. May also be dissolved either in 0.8% w/v  $\text{NaHCO}_3$  solution or in Tris hydrochloride (Tris HCl).

#### **Streptozotocin**

It is dissolved in ice-cold 50 mM sodium citrate buffer (pH 4.5) containing 0.9% NaCl immediately before injection.

#### **Apomorphine sulphate**

Dissolved in 0.9% NaCl containing ascorbic acid 0.2 mg/ml.

#### **Cimetidine**

Dissolved in small quantity of 0.1 N HCl neutralized by 0.1 N NaOH, and made up to a volume with 0.9% NaCl. May also be prepared in saline as 2% suspension.

#### **L-Dopa HCl**

Dissolved in 0.1 N HCl to give a concentration of 40 mg/ml.

#### **Disulfiram**

Suspended in solution using compound powder of tragacanth.

#### **FLA-63**

Dissolved in hot dilute 1% tartaric acid.

#### **Kainic acid**

Dissolved in 0.5 mM phosphate buffered saline.

#### **Metiamide**

Same as for cimetidine.

#### **Metergoline**

Suspended in 5% carboxymethylcellulose.

#### **Nifedipine**

Dissolved in 30% ethanol.

### Niludipine (Nisoldipine)

Dissolved in 5% polyethylene glycol.

### Oestradiol

Dissolved in 3 parts of absolute ethanol and 7 parts of saline.

### Spiroperidol (Spiporone)

Dissolved in a few drops of glacial acetic acid, and made up with distilled water; pH adjusted to 6.0.

### Heparin

It is available as sodium salt containing 100 units per mg; used intravenously in a dose (units per kg) 500 in dog, 1000 in cat, 2000 in rat, and 3000 in rabbit. In order to prevent clotting in arterial cannula, 0.5 ml of 1% solution (500 units) of heparin is injected into the cannula before the arterial clamp is removed. In rat, a polyethylene cannula is filled with 0.1% solution of heparin. In order to minimize the *in vitro* formation of pharmacologically active substances, it is the only suitable anticoagulant for blood. For such purposes, 1 vol. of a 1% pure heparin sodium in 0.9% NaCl is added to 20 vol. of blood.

### Anticoagulant Fluid

Sodium citrate (8.5%), sodium thiosulphate (25%) or half-saturated (or 30%) sodium sulphate solution (filtered) is used as an anticoagulant fluid to fill the tubing connecting the manometer with the arterial cannula for the recording of blood pressure. To prevent clot formation in the cannula the following procedure may also be adopted. The cannula is first rinsed with 2% silicone in carbon tetrachloride, dried in an oven and then well rinsed with tap water leaving a non-wettable film of silicone on the surface. Heparin 0.25 ml of 1% solution is then placed in the tip.

## SOLVENTS OR VEHICLES

Dilute hydrochloric acid, dilute sodium hydroxide, or 5% gum acacia suspension are used as solvents for test substances.

Propylene glycol is a useful solvent of low toxicity for substances which are insufficiently soluble in water, or are unstable in aqueous solution. It may be used for injections, although it might have some local irritant effect by intramuscular route.

Polysorbate (Tween 80) is a non-ionic surface-active agent used as a solubilizing agent for water insoluble substances for injection. Although it is claimed to be an inert substance without any pharmacological action, some actions have been



reported in the form of histamine release in the dog, and spasmolytic effect on isolated tissues (Sabir *et al.* 1972).

Sesame oil is one of several solubilizing agents, which are used in the animal for testing of water insoluble drugs such as  $\Delta^9$ -tetrahydrocannabinol (THC). It was found to reduce noradrenaline in the brain, heart and spleen of mice. This indicates that all solubilizing agents are not necessarily pharmacologically inert (Anton *et al.* 1974).

PREPARATION OF APPROXIMATE  
NORMAL SOLUTIONS

<i>Acid or Alkali</i>	<i>ml diluted to 1l with distilled water</i>
HCL	100
HNO <sub>3</sub>	63
H <sub>2</sub> SO <sub>4</sub>	28
NaOH	42 g

ATOMIC WEIGHTS OF SOME COMMON ELEMENTS

H	C	N	O	F	Na	Mg	P	S	Cl	K	Ca	Fe	I
1	12	14	16	19	23	24.3	31	32.1	35.5	39.1	40.1	55.9	126.9

SOLUBILITY

<i>Description</i>	<i>Relative quantities of solvent for 1 part of solute</i>
Very soluble	Less than 1 part
Freely soluble	From 1 to 10 parts
Soluble	From 10 to 30 parts
Sparingly soluble	From 30 to 100 parts
Slightly soluble	From 100 to 1,000 parts
Very slightly soluble	From 1,000 to 10,000 parts
Practically insoluble	More than 10,000 parts

STORAGE TEMPERATURE

Cold place - having a temperature not exceeding 15°C

Refrigerator - a cold place in which the temperature is held between 2°C and 15°C

Excessive heat - temperature above 49°C

Freezing of biological products – provided container remains intact, freezing does not affect generally the potency of biological products.

### RELATIVE SIZE OF MOLECULES AND CELLS

Molecular weight of any compound expressed in grams contains  $6.02 \times 10^{23}$  molecules. Thus, a compound with a molecular weight of 200 :

200 g contain about  $6 \times 10^{23}$  molecules

2 mg contain about  $6 \times 10^{18}$  molecules

1 mg contains about  $3 \times 10^{18}$  molecules

### UNIT AND MEASURES

Quantity	Base Unit	Symbol
Mass	gram	g
Concentration	mole	mol
Volume	litre	l
Length	metre	m
Time	second	s
Temperature	Celsius	°C
Current	ampere	A
Frequency	hertz	Hz
Radioactivity	curie	Ci
Pressure	millimetre of mercury	mmHg

### PREFIXES FOR VARIOUS UNITS

mega	(M)	-	$10^6$
kilo	(k)	-	$10^3$
milli	(m)	-	$10^{-3}$
micro	( $\mu$ )	-	$10^{-6}$
nano	(n)	-	$10^{-9}$
pico	(p)	-	$10^{-12}$
femto	(f)	-	$10^{-15}$
atto	(a)	-	$10^{-18}$

### FURTHER READING

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 Luduena, F.P. (1960) In *Methods in Medical Research*, ed.-in-chief Bruner, H.D., vol.8, Year Book Publishers, Chicago.  
 Sabir, M., Singh, M. and Bhide, N.K. (1972) *Ind. J. Physiol. Pharmacol.* 16, 193.  
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# Some Basic Equipments

## ASSEMBLY FOR RECORDING OF THE BLOOD PRESSURE

The mercury manometer originally designed by Poissuille in 1828 was modified by Carl Ludwig in 1847 to allow graphic records to be obtained from a float on the mercury, so that progressive changes in the blood pressure could be studied. Even today, the assembly virtually remains the same, consisting of a mercury manometer with a float supporting a long stiff wire that writes on the smoked surface of the kymograph with the help of a stylus. Owing to the inertia of the column of mercury, the assembly does not, however, register accurately the rapid changes of pressure in the artery with each heart beat that appears as comparatively small fluctuation. These fluctuations become larger when the heart beat is slow, because the manometer is now capable of keeping up with the slow rate. The assembly thus gives only a true and valuable record of the mean arterial pressure.

The mercury manometer consists of a glass U-tube (5 mm bore) with two vertical limbs about 30 cm in height, which is half-filled with mercury. Since the mercury is displaced equally up in one limb and down in the other, it is obvious that any displacement recorded must be multiplied by two to obtain the actual pressure in terms of a mercury column. To obviate this, a millimeter scale with doubled values is fitted with the manometer so that these can be read off directly up to 250 mmHg. On the surface of the mercury column in one limb is a cylindrical float of vulcanite from which a stiff fine wire rises bearing on its upper end a stylus (writing point) that writes on the traveling surface of the smoked paper. The other limb of the manometer has a side-tube that is connected through inextensible (*pressure or resistance*) tube made of thick rubber or polythene to an arterial cannula. The upper end of this limb is also connected with a reservoir bottle containing some anticoagulant fluid that can be pumped into this limb and through the interconnecting tube to the arterial cannula. At the junction of the side-tube, a 3-way stopcock connects or disconnects the arterial cannula and the manometer from the reservoir bottle (Fig. 5.1).

*Condon's blood-pressure manometer.* It is an assembly for recording of the blood pressure in rats and other small animals, and differs from the conventional manometer in the following respects. The main limb of the manometer is 28 cm long and the bore 2.5 mm in diameter. A reservoir 2.5 cm diameter replaces one of the limbs so that the mercury level falls but little in the reservoir when the column of mercury rises in the small bore of the main limb, thus giving approximately double the sensitivity of the normal U-shaped manometer. The scale is calibrated

## Some Basic Equipments

to take this small change into account so that accurate pressure readings between 0 and 200 mmHg may be obtained.

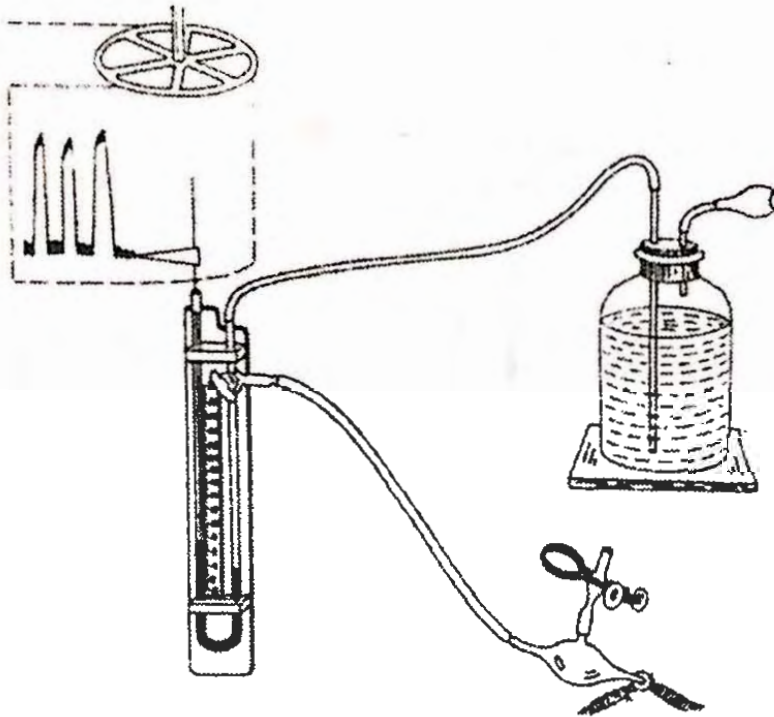


Fig. 5.1. Assembly for recording of the blood pressure in anaesthetized animal. For details see text.

### ASSEMBLY FOR RECORDING OF CONTRACTION OF ISOLATED TISSUES

Rudolph Magnus was the first to design the arrangement of bath for excised organs (intestinal strips) as early as in 1904. Even today the assembly remains basically the same consisting of (a) a water bath made of glass or Perspex fitted with electrical heater with thermostatic control, and a small electrical stirrer to keep the water in the bath circulating for uniform heating, (b) a cylindrical organ bath made of glass of variable capacity (5 to 100 ml) for suspending the tissue in physiologic salt solution, (c) a coil made of glass or Perspex connected to the lower end of the organ bath by means of a short length of rubber tubing to keep the salt solution warm before entering the organ bath, (d) an oxygen tube cum tissue holder made of glass, (e) a writing lever, and adjustable clamps, holders, grips, etc. for holding the oxygen tube, writing lever and thermometer in position. A diagrammatic sketch of the set up is presented in Fig. 5.2.

The contractions and relaxations are recorded with the help of the writing

lever on a smoked paper fixed round an electrically operated recording drum, which can be adjusted to any desired speed depending on the nature of the experiment. For most of the experiments a speed of one revolution in 96 minutes is usually used.

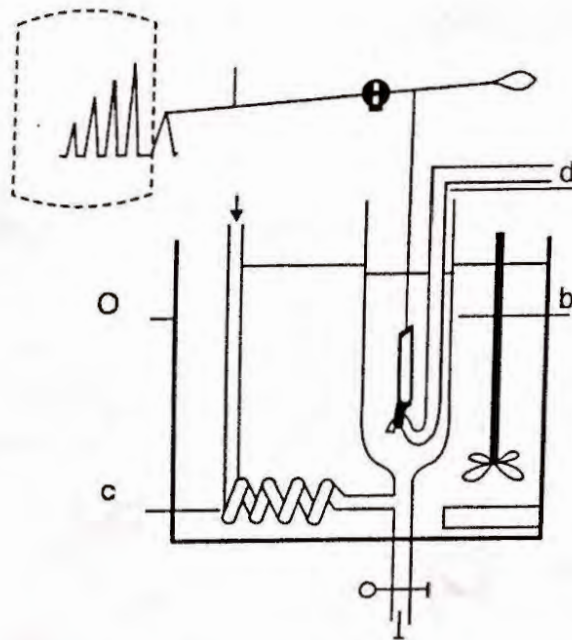


Fig. 5.2. Assembly for recording of contractions of isolated tissues. For details see text.

### The Writing Lever and its Adjustments

The levers mostly used in the isolated tissue work are made of aluminium, stainless steel, balsa wood, or sometimes of 'drinking straw' that are light and rigid, so that while writing on a smoked surface they do not bend. Most of the writing levers belong to the 'class I type' of mechanical levers, i.e. the fulcrum or pivot lies between the writing point and the point of attachment of the tissue. Starling's heart lever, however, belongs to the 'Class II type' of lever since the fulcrum lies at one end beyond the point of attachment. The levers are usually of *isotonic* type, i.e. the change in length due to contraction is recorded, while the tension on the muscle remains the same. *Isometric* levers, which record the change in tension only, are also used under special circumstances. For instance, when a twitch is produced by stimulating a muscle suspended between two rigid points, one being a strong spring; the muscle does not shorten, but only creates a force or tension that is recorded; the twitch is also much faster in action. For studying the relationship between concentration of a substance and the effect on an isolated tissue, it is preferable to work over as wide a range of concentration as possible. For this, measurement of force rather than of contraction is preferable, since the former has no limit, whereas a muscle can shorten (contract) only so much. For

## Some Basic Equipments

delicate tissues, an isometric strain-gauge transducer should be ideal; alternatively, Paton's auxotonic lever (see later) will serve the purpose.

*Adjustment for magnification of recording.* The fulcrum should be so placed that there is some magnification of the actual contractions. In order to achieve this, the distance between the writing point and the fulcrum (F) should always be greater than that between the point of attachment to the tissue (T) and the fulcrum. By adjustment of these relative distances from the fulcrum, any degree of magnification can be obtained. For instance, in Fig. 5.3, if the distance A is 10 cm and B 2 cm, then the magnification is obtained by the ratio  $A/B = 5$ . The magnification to be employed is dependent on the individual tissue (see Table 17.1). Lesser the inherent contractility of a tissue, higher the magnification needed, and *vice versa*. For instance, guinea pig ileum requires five to ten times magnification, while the uterus requires only about one and a half times.

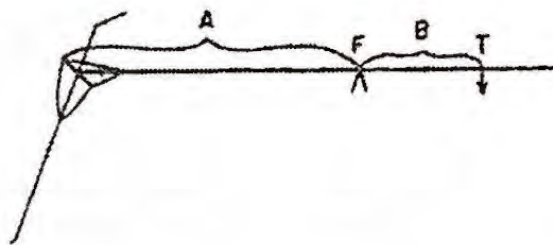


Fig. 5.3. Adjustment for magnification of recording. For details see text

*Adjustment of load or tension.* Since the fulcrum is always away from the midpoint, the lever is first balanced by attaching sufficient weights (W) on the shorter arm near the end. For efficient contractions, enough tension is applied on the muscle so that when unstimulated, it relaxes to its full extent, but not so as to obliterate its normal tone, or spontaneous rhythmic movements. This is achieved by either of the two ways: (a) after balancing the lever (with W), the effective load (L) for the particular tissue is applied to the longer arm of the lever at the same distance from the fulcrum as the point of attachment of the tissue (T) (Fig. 5.4a), or (b) the lever is first balanced by hanging small weights equivalent to the effective load for the particular tissue at the point of attachment of the tissue (L), and also by

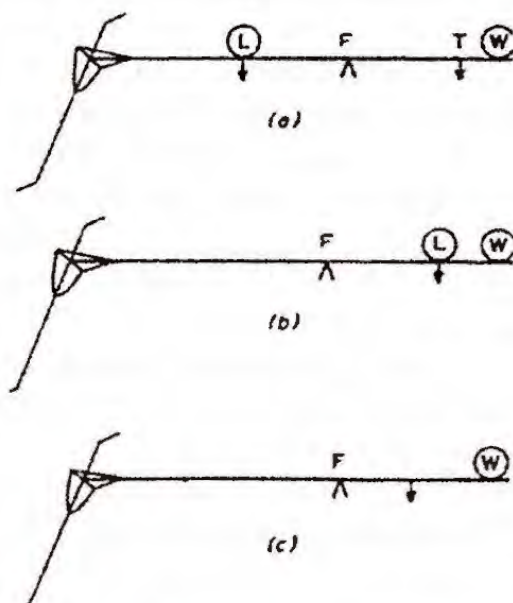


Fig. 5.4. Adjustment of load or tension of writing lever. For details see text.

putting remainder of the weights at the end (W) (Fig. 5.4b); the weights equivalent to the effective load are then removed from the point of attachment of thread (Fig. 5.4c).

In the case of tracheal chain of the guinea pig, where the tension should be minimal, the weights equal to that of the chain is removed. Thus the only tension used in this case is that caused by the weight loss of the chain through the buoyancy of the bath fluid. Hooks with weights are available for the purpose, but balls of plasticine (modelling clay) of different weights may serve the purpose well.

### Different Types of Lever

*Simple lever.* It consists of light aluminium, or stainless steel capillary fitted through an axle pivoted between hardened centres mounted on a stout brass frame; a thumbscrew located on the axle midway between the centres permits the lever to be adjusted for length as required. A light celluloid writing point (stylus) is fitted at one end that gives a tangential record on the smoked drum.

*Frontal writing lever.* It is so designed that the actual shortening of the muscle is magnified in linear proportion on the drum. The lever is made of a strip of aluminum fixed to a wheel that rotates freely about its axle. A long frontal-writing point made of glass capillary is attached to the end of the strip by means of a hinge.

*Heart lever (Starling's).* It consists of a frame carrying a light lever arm with holes and notches supported by a fine adjustable nickel-silver spring attached to an adjustable hook.

*Universal lever (Brodie's).* It is a general utility lever with axis screwed with two nuts between which the interchangeable levers are clamped. It is complete with one plain lever, one notched lever with holes, and one short lever.

*"Fixit" lever.* It is similar to the Brodie's, but the central spindle has an ebonite roller on which three pins are set at right angles. There is a rod carrying a spring, which slides in the frame underneath the roller, so that the lever can be held in any desired fixed position. This is usually operated through camera shutter release, and is particularly useful to prevent the upward kick by the lever during washing.

*Gimbal lever.* With this lever the pressure of the stylus is effected by the force of gravity, thus obviating adjustments necessary in the case of ordinary levers.

*Isometric lever.* Consists of a heavy brass frame with spring steel wire fixed to each limb; the lever passes through the centre of the spring. A screw is so arranged that it forces the limbs apart and increases the tension of the spring.

*Pendulum auxotonic lever (Paton's).* An auxotonic lever is a light spring-loaded lever in which the load on the muscle increases as it shortens. The lever becomes virtually isotonic when the slope of the line relating the load of deflexion is low enough. Thus, with this lever both the shortening and the tension increase with increasing contraction. A simple way of making an auxotonic lever is to attach a pendulum to the lever. A small perforated aluminium strip is mounted downwards at right angles to a frontal lever made with perforated aluminium strip, and bent

at a convenient distance from the fulcrum to make a small support to which weights may be attached (Paton, 1957).

### MULTICHANNEL POLYGRAPH RECORDERS

These are highly sensitive direct writing oscillographic recorders intended for measuring and recording different physiological events, such as blood pressure, muscle contractions (both isotonic and isometric), temperature, etc. Most of the recorders consist of three components:

1. *Transducer.* A device that receives the input signals in the form of any energy (e.g. muscle contraction, respiration, pressure and temperature changes, etc.) and converts it to an electrical energy.
2. *Amplifier.* Most of the commercial recording units usually provide voltage amplification, so that a deflection of 2 cm at the output can be caused by 1 mV at the input. Since the commonly recorded biologic potentials lie between 50 mV and 150 mV, an additional multiplication of the biologic signal by 100 in a pre-amplifier provides an adequate range of amplification for most of the experiments. Pre-amplifier thus gives the signal an initial boost, and a power driver (amplifier) amplifies further the pre-amplifier signal so as to drive the pen motor through a galvanometer.
3. *Recording system.* A chart-drive device that moves the chart paper at a variable speed.

Many models with capacity up to eight channels are available (Grass Polygraph; Beckman Dynograph; Inco Polyrite; Encardiorite), so that a simultaneous recording of a number of events can be obtained. In addition to the recording channels, time and event channels are also provided in these instruments. By using different pre-amplifier modules and appropriate transducers, recordings can be obtained for different events.

### ELECTRONIC STIMULATOR

It provides square waves (rectangular pulses) of current of varying duration, frequency and voltage (Fig. 5.5) required for most of the investigations. The output voltage required depends on the tissue that is to be stimulated. Thus, frog nerve

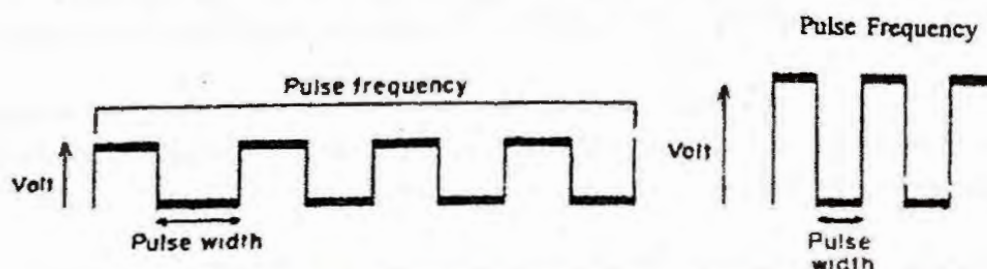


Fig. 5.5. Square wave or rectangular pulses.



needs only one or two volts, whereas stimulation of human nerve through the skin may need 50 volts or more. Pulse duration of 1 or 2 ms is quite suitable for stimulation of the nerve. Long pulses of current are best suited for stimulation of smooth muscles or of sympathetic nerves. A *supramaximal voltage* is obtained by increasing the voltage until there is no further increase in the height of contraction, and then setting the voltage to about 50% greater than this. To avoid current spread, the cervical sympathetic nerve placed on a shielded bipolar platinum electrode is immersed in liquid paraffin.

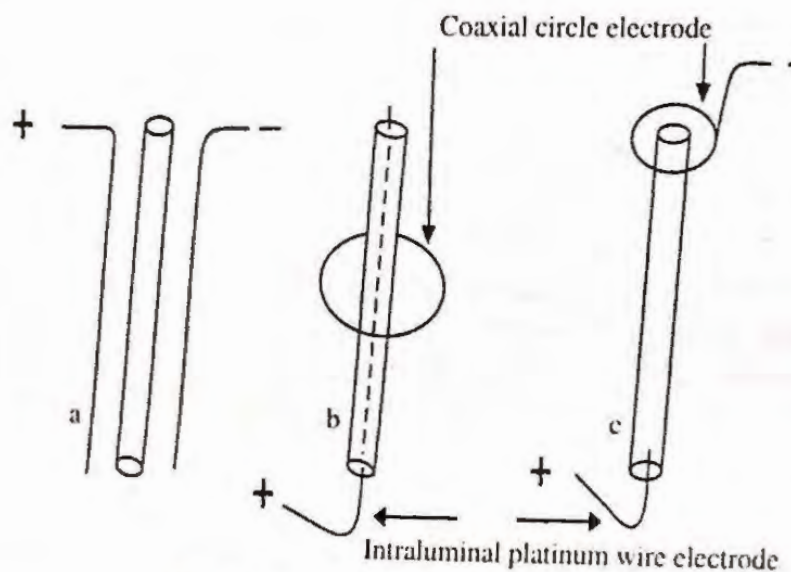


Fig. 5.6. Arrangements for transmural (electrical field) stimulation. For details see text.

*Transmural (electrical field) stimulation.* It is used to excite the intramural nerves of isolated tissues in one of the following ways:

- i) Two lengths of platinum wire are cemented to the edges of a Perspex gutter, so that they are held parallel and about 4 mm apart (Fig. 5.6a). The isolated tissue is suspended between the two wires in a bathing fluid.
- ii) One platinum wire is threaded inside the lumen of the viscus (intraluminal electrode) as one electrode with a coaxial circle of wire as the other electrode (Fig. 5.6b).
- iii) A short length of platinum wire just enters the lumen at the lower end as one electrode with a coaxial circle of wire at the upper end as the other electrode (Fig. 5.6c).

The details of the nature of stimulation to be applied to different nerve and tissue preparations are given in Table 5.1

Table 5.1  
Stimulation of different tissues by square wave pulses

Tissues	Strength (V)	Duration (ms)	Frequency (Hz)	Stimulation (period)
Dog				
Vagus nerve (peripheral)	10	5	8	
Cat				
Nictitating membrane through cervical sympathetic nerve	Supramaximal "	0.5 0.5	10 6	15 sec every 2 min 6 sec
	15	2	5	10 sec
Splanchnic, vagal, superior cervical nerve	Submaximal or supramaximal	1	5-30	10 sec every 2-3 min
Rabbit				
Jejunum innervated (Finkelman) preparation	10 Supramaximal (15-25)	0.5 2	50 5-50	20 sec every 7 min
Guinea pig				
Atria	Twice threshold Maximal Supramaximal	0.5 5 2	2 1 3	
Ileum	Maximal / (transmural stimulation) Supramaximal (40)	supramaximal 1.3 × maximal 0.5	0.4 or 1 0.5 0.05	0.1 0.1
Taenia caecum	40-50	0.2	30	30 sec
Vas deferens (transmural stimulation)	Supramaximal 90-120	0.1 0.5	25 5-50	15 sec every 3 min 5-45 sec every 5 min
Hypogastric nerve-vas deferens	Supramaximal or Submaximal (2-3)	1-2	50-80 every 2 min or for 2-5 sec every min	100 or 200 pulses
Rat				
Fundal strip	Supramaximal (15)	1	1-10	10 sec every 2 min
Phrenic nerve-diaphragm	Submaximal Supramaximal (1-4)	1 0.2	0.1 0.2-0.3 by 50 Hz for 10 sec	Tetanus produced
Direct stimulation of diaphragm	20-60	1	0.2	
Vas deferens through nerve	Supramaximal	1	2	20 sec every 3 min

Table 5.1 (Cont.)

Tissues	Strength (V)	Duration (ms)	Frequency (Hz)	Stimulation (period)
Vas deferens field stimulation (mounted between longitudinal platinum electrodes)	10-30 Supramaximal 80 - 120	3 1 1	0.1 5 20	10 sec every 4 min
Anococcygeus muscle	Supramaximal	1	10	15 sec
Atria	0.3-1.5 (slightly above threshold)	3	1	
Mouse				
Vas deferens (field stimulation)	1.3 × maximal Supramaximal	0.5-2 0.3	0.1 30	every 3 min

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# Pharmacology of Neurohumoral Transmission and Sympathectomy

## NEUROHUMORAL TRANSMISSION

Broadly two steps are involved in the transmission of a nerve impulse along a nerve path: (a) *conduction* or passage of an impulse along an axon, and (b) *transmission* or passage of an impulse across a synaptic or neuroeffector junction.

### Axonal Conduction

In response to a stimulus above the threshold level, a nerve *action potential* (AP) or nerve impulse is initiated. A series of electrical and ionic changes in the membrane bring about a propagation or conduction of AP without decrement along the axon. The axonal conduction can be blocked by a few toxins, e.g. tetrodotoxin, batrachotoxin, etc., the mechanisms of which have been summarized in Table 6.1

Table 6.1  
Axonal conduction blockers

<i>Toxin</i>	<i>Source</i>	<i>Mechanism</i>
Tetrodotoxin Saxitoxin	Puffer fish Dinoflagellates eaten by clams and other shell-fish	Prevent rapid inward Na <sup>+</sup> current leading to blockade of propagated action potential in nerve and muscle.
Batrachotoxin	Steroidal alkaloid secreted by a South American frog	Selective increase in Na <sup>+</sup> permeability leading to persistent depolarization and blocking of axonal conduction
β-Bungarotoxin*	Venom of <i>Bungarus</i> <i>multicinctus</i>	Blocks neuromuscular transmission presynaptically

\* α-Bungarotoxin acts postsynaptically

### Junctional Transmission

The arrival of the AP at the axonal terminals initiates a series of events in succession as follows:

1. *Release of the transmitter.* The depolarization of the axonal terminal triggers the release of a large quantity of neurotransmitter synthesized and stored in the nerve terminals.
2. *Combination of the transmitter with post-junction receptors, and production of the post-junctional potential.* The transmitter diffuses across the synaptic or junctional cleft, combines with postsynaptic receptors resulting generally in a localized nonpropagated increase in the ionic permeability, or conductance of the membrane. A generalized increase in permeability to all types of ions results in a localized depolarization of the membrane, that is, an *excitatory postsynaptic potential (EPSP)*, while a selective increase in permeability to only potassium and chloride results in stabilization or actual hyperpolarization of the membrane, that is, an *inhibitory postsynaptic potential (IPSP)*.
3. *Initiation of post-junctional activity.* If an EPSP exceeds a certain threshold value, it initiates propagated AP in a neuron, or a muscle AP in cardiac and skeletal muscles, or a localized contractile response in smooth muscles, or secretion by gland cells. An IPSP tends to oppose excitatory potentials initiated by other neuronal sources at the same site.
4. *Destruction or dissipation of the transmitter.* The transmitter is rapidly removed following each impulse by one or more of the following mechanisms:
  - (a) enzymic destruction,
  - (b) re-uptake into neuronal or extraneuronal sites, and
  - (c) simple diffusion.

### IDENTIFICATION OF A SUBSTANCE AS A NEUROTRANSMITTER

Before a substance (putative transmitter) can be designated as 'neurotransmitter', it has to satisfy the following criteria:

1. Demonstration of its presence along with its precursors in the appropriate nerve endings.
2. Enzymes or systems for its synthesis and breakdown should be demonstrable in the nerve.
3. Recovery of the substance from the perfusate by stimulation of the nerve.
4. Its administration should mimic the action produced by appropriate nerve stimulation.
5. Modification by drugs of responses produced by both nerve stimulation and administration of the putative transmitter.

### SYMPATHECTOMY

Sympathectomy is an experimental procedure employed to assess the role of peripheral sympathetic nervous system in various physiological processes, and to elucidate the mechanism of action of certain drugs.

#### SURGICAL SYMPATHECTOMY

In this procedure, sympathetic nerve supply to an organ is cut, e.g. denervation of the nictitating membrane of cat by removal of the superior cervical ganglion eight days previously. The drawback is that the period of denervation is brief, since reinnervations by adrenergic nerves from neighbouring tissues and organs occur soon after denervation..

#### IMMUNO SYMPATHECTOMY

Injection of the *nerve-growth factor (NGF) antiserum* in newborn rats destroys more than 90% of sympathetic cell population (paravertebral and coeliac ganglia). NGF, a protein isolated in pure form from the male mouse salivary glands, is essential for the development of sympathetic nerve cells. Higher levels of NGF have been found in guinea pig prostate gland that may serve as a rich source of NGF (Harper *et al.* 1979). The drawbacks of immunosympathectomy are (i) difficult to raise NGF antiserum, (ii) incomplete sympathectomy, (iii) both afferent and efferent nerves are affected, and (iv) ineffective in adult animals.

#### CHEMICAL SYMPATHECTOMY

##### 6-Hydroxydopamine (6-OHDA)

This produces (a) *reversible changes* in adult animals, selectively destroying peripheral adrenergic nerves alone leaving the cell body intact, thus providing the possibility for subsequent regeneration, and (b) *irreversible changes* in newborn animals where the whole neuron is destroyed.

The accumulation of 6-OHDA in the adrenergic neuron by the neuronal amine pump seems to be a prerequisite for its destructive action on the nerve. This transport mechanism, which is present in the cell body, gets shifted to the axons of the sympathetic neuron during adulthood. This explains the difference in the action of 6-OHDA in adults and in newborns.

Injection of large dose of 6-OHDA into the brain can produce a selective destruction of noradrenergic and dopaminergic nerve terminals in the brain. The drawbacks are : (i) regeneration of the axons occurs in a few weeks, as the cell bodies are not markedly destroyed, (ii) vascular adrenergic nerve terminals regenerate more rapidly than the nonvascular nerves, and (iii) heart and large vessels are affected first, then the peripheral vessels, and lastly the genital system.

## Guanethidine

This produces widespread sympathectomy even in adult animals, and has the following advantages : (a) the effect persists over long period (permanent sympathectomy), (b) particularly suitable for studies of the reproductive organs which are denervated effectively, (c) produces complete sympathectomy of the vascular smooth muscles, and (d) does not affect central neurons in the brain.

### CHEMICAL DESTRUCTION OF TRYPTAMINERGIC NEURONS

Neurotoxins that are preferentially taken up into tryptaminergic neurons, thereby destroying these neurons are 5, 6- and 5, 7-dihydroxytryptamines.

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# Pharmacology of Receptors

As early as late nineteenth and early twentieth centuries, Ehrlich and Langley introduced independently the original concept of receptor sites. During the past few decades, progress had been made in the isolation and characterization of receptors by radioligand binding techniques. It is now well established that the receptor is a cellular macromolecule, or an assembly of macromolecules, mainly protein in nature, present on the surface of the cell membrane or inside a cell, concerned directly and specifically in chemical signaling between and within the cells. When the endogenous agonists like hormone, neurotransmitter or intracellular messenger, and exogenous agonists like drugs and chemicals get attached with the receptor(s), there is a change in the cell function producing a response.

## Structure and Properties of Receptors\*

The regions of the receptor macromolecule to which ligands\*\* bind are known as the *recognition sites*, consisting of *primary or orthosteric sites* to which the endogenous agonist binds, and *allosteric or allotropic sites* to which the other ligands bind. Agonists may act by combining either with the primary or orthosteric sites, or less commonly, with the allosteric or allotropic sites.

*Allosteric agonists or activators* are ligands that are able to mediate receptor activation on their own by binding to a recognition site on the receptor macromolecule that is different from the primary site.

*Allosteric enhancers or modulators* enhance the orthosteric ligand affinity and/or agonist efficacy, while having no effect on their own.

*Allosteric antagonists* are modulators that reduce the orthosteric ligand affinity and/or agonist efficacy. *Neutral allosteric ligands* bind to an allosteric site without affecting the binding or function of orthosteric ligands, but can still block the action of other allosteric modulators that act via the same allosteric site.

*Syntopic interaction* is a competitive interaction between the ligands that bind to the same (common) orthosteric or allosteric site on the receptor macromolecule.

*Allosteric interaction* is a cross-interaction between different sites on a receptor macromolecule.

There are four broad families of receptors as follows:

- (a) *Ionotropic (channel-linked) receptors* – are linked directly to an ion channel which opens only when the receptor is occupied by an agonist (ligand-

\* Neubig, et al (2003) \*\*Ligand is a molecule (agonist or antagonist) that binds to a receptor protein.



gated ion channels) producing depolarization and increase in cytosolic  $Ca^{2+}$ .

- (b) *Metabotropic (G protein-coupled) receptors (GPCRs)* – a large family of receptors that serve as sites of action for many drugs. They are so named because their activity is regulated by interaction with guanine nucleotide regulatory protein, G protein. Agonists binding to these receptors promote the dissociation of the guanosine diphosphate (GDP), and binding of guanosine triphosphate (GTP) to the G protein that leads to the dissociation of the GTP bound alpha subunit, which in turn combines with the effector enzyme systems generating the second messenger leading to a response. Calcium sensing receptor (CaR) belongs to the superfamily of G protein-coupled receptors. The CaR controls extracellular calcium homeostasis via parathyroid hormone (PTH) secretion and renal calcium reabsorption. The CaR selective calcimimetic drug Cinalect stimulates the CaR to suppress PTH secretion in chronic kidney disease.
- (c) *Kinase-linked receptors* – contain a protein kinase domain within the cell with intrinsic enzymatic activity.
- (d) *Receptors that regulate gene transcription (nuclear receptors)* – are soluble cytosolic or intracellular proteins.

A receptor exists in two forms, active ( $R_2$ ) and inactive ( $R_1$ ), whose properties are presented in Table 7.1

Table 7.1  
Properties of active ( $R_2$ ) and inactive ( $R_1$ ) receptors

Properties	Active Receptor ( $R_2$ )	Inactive Receptor ( $R_1$ )
Ion channel	Open	Closed
Protein tyrosine kinase	Active	Inactive
Coupling to G protein	Productive	Non-productive
Affinity for full agonist	High	Low
Affinity for partial agonist	Intermediate	Low
Affinity for antagonist (competitive)	Equal for both receptors	
Affinity for inverse agonist	Low	High

The following forces that allow interaction between the functional groups of the drug and the complementary binding surfaces on the receptors determine the binding of a drug to a receptor:

- Hydrogen bonds
- Ionic bonds
- van der Waals forces
- Covalent bonds

When a ligand or a drug binds to a receptor, a complex is formed to produce a

stimulus leading to a chain of biochemical effects in the cell (second messengers), ultimately producing a response. Second messengers, such as cyclic AMP, cyclic GMP,  $\text{Ca}^{2+}$ , inositol phosphates, diacylglycerol and nitric oxide influence one another, both directly by altering their metabolism and indirectly by sharing intracellular targets. Cyclic AMP is synthesized by adenylyl cyclase under the control of G protein-coupled receptor, stimulation of which is mediated by  $G_s$  and inhibition by  $G_i$ . Cyclic AMP functions by activating cAMP - dependent protein kinases, which in turn phosphorylate physiological targets (metabolic enzymes or transport proteins), numerous protein kinases, and other regulatory proteins. It also directly regulates the activity of plasma membrane cation channels.

### Agonist

It is a ligand capable of binding with receptors so as to alter the receptor state thereby eliciting a response. Two factors determine the ultimate response of a tissue, (i) *affinity*, that describes the strength with which an agonist combines with the receptor, and (ii) *intrinsic activity* or *efficacy*, that is the ability to produce a response once the receptors are occupied by the agonist. The activity of an agonist depends on the product of affinity and intrinsic activity. Affinity and intrinsic activity are independent properties of ligands. Agonists have both affinity and intrinsic activity. The relative intrinsic activities are expressed for compounds that interact with the same specific receptors producing the same type of response. Ariens introduced the term  $\alpha$  for intrinsic activity, which may vary from 1 for a full (pure) agonist to 0 for a pure competitive antagonist.

*Full (pure) agonist* elicits maximal response as it has an intrinsic activity  $\alpha$  equal to unity.

*Partial agonist* having intermediate intrinsic activity  $\alpha$  between 0 to 1 combines with the receptors eliciting only a submaximal response even in higher concentration. Its position is somewhere between pure agonist and pure antagonist. Partial agonist may also act as a partial antagonist. For instance, nalorphine, a partial agonist, produces weak morphine-like effects, while antagonizing the effects of morphine.

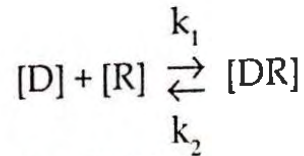
*Inverse agonist* combines with the receptors producing reduced receptor activity.

### Antagonist

A *ligand* that combines with the receptor without producing any response but inhibits the effect of an agonist by impairing the formation of agonist - receptor complex. An antagonist has affinity but no intrinsic activity. Selective antagonists have relatively higher affinities for receptors than that of even potent agonists. Many antagonists are receptor specific in low concentrations, but lose their specificity in higher concentrations thereby blocking a number of other receptors.

## RECEPTOR THEORIES OF DRUG ACTION

*Occupation theory.* According to the classical occupation theory of Clark, a response to an agonist is some positive function of the number of receptors occupied by the agonist. The binding of a drug (D) to the receptor (R) can be described by the following expression:



where [D] is the drug concentration, [DR] the drug-receptor complex,  $k_1$  the rate of association of the drug with receptor, and  $k_2$  the rate of [DR] complex dissociation. At equilibrium, the amount of DR formation is equal to the amount of DR dissociation, and thus the steady state amount of [DR] is constant:

$$[D][R] k_1 = [DR] k_2$$

$$k_1/k_2 = [DR] / [D][R]$$

Affinity is the ratio of  $k_1$  over  $k_2$  ( $k_1/k_2$ ). The reciprocal of affinity ( $k_2/k_1$ ) that is  $K_d$  is the equilibrium dissociation constant. The dissociation constant  $K_d$  is a useful measure of concentration (usually micromolar or nanomolar) which describes the strength of binding (affinity) between receptors and their ligands. It is unique for each ligand (drug) for respective receptor system; hence can be used to identify receptors. There is an inverse relationship between the  $K_d$  and affinity. The smaller the  $K_d$ , the greater the affinity, and *vice versa*. The degree of receptor occupancy is dependent upon the concentration of the ligand as well as its affinity constant. In general, ligands with high affinity constant will occupy more receptors at any given concentration than those with low affinity constant.

*Rate Theory.* According to Paton's rate theory, response is a function of the rate of association between drug molecules and receptors. Following an association, more occupation does not produce any further response. As a matter of fact, the receptor is not free for another association until it is unoccupied. For equilibrium conditions, drugs with very high dissociation constant ( $k_2$ ) will be strong agonists, those with very low  $k_2$  will be essentially pure competitive antagonists, and those with  $k_2$  values in an intermediate range will be partial agonists.

## EXPERIMENTAL METHODS FOR CHARACTERIZATION OF RECEPTORS

*Variants of Receptors (Isoreceptors).* A receptor might have a number of agonists in common, although their order of potency may be different. In order to discriminate between variants of certain receptors, relative potencies of a series of agonists and antagonists can be compared. If the receptor sites are identical, the order of potency must be the same; if the order of potency differs, the receptors must be different. On the basis of such studies, Ahlquist in 1948 postulated two types of adrenergic receptors, viz.,  $\alpha$  receptors and  $\beta$  receptors. He found that the

order of potency on smooth muscle producing contraction was adrenaline > noradrenaline >> isoprenaline, which he designated as  $\alpha$ -adrenoceptor effect. The order of potency on smooth muscle producing relaxation was isoprenaline > adrenaline >> noradrenaline, which he designated as  $\beta$ -adrenoceptor effect. The order of potency for stimulation of the heart was similar to that for relaxation of smooth muscle, that is  $\beta$ -adrenoceptor effect.

The classification and sub-classification of receptors are based mainly on the application of one or more of the following techniques:

### 1. Relative Potency (Affinity) Measurements of a Series of Agonists

Concentration-response curves on a number of tissues or organs representing different receptor systems under ideal conditions (*see later*) are obtained for a series of agonists, such as adrenaline, noradrenaline, phenylephrine and isoprenaline, and the relative potencies of these agonists are compared. The receptor type can be identified by inspection of these data tabulated for different tissue receptors. Furchgott (1967), for instance, observed a similar potency series (adrenaline > noradrenaline > phenylephrine » isoprenaline) when tested on three different tissues of the rabbit (thoracic aorta, stomach and duodenum) suggesting that the  $\alpha$  receptors in these tissues are essentially of one type. By calculating the correlation coefficients of the relative potencies, a correlation between two systems may suggest a similar receptor type. For instance, correlation coefficient of sympathomimetic amine activity on receptor systems 'bronchodilatation-vasodepression' was 0.96, while that of 'cardiac stimulation-bronchodilatation' was 0.31, suggesting the former as a single receptor system ( $\beta_2$ ), while the latter different receptor systems (Lands *et al.* 1967).

The selectivity ratio (*see later*) between different tissues or organs may be utilized for the classification of receptors into subtypes. As an illustration, clonidine has most selective effect on presynaptic  $\alpha_2$  adrenoceptors, while phenylephrine on postsynaptic  $\alpha_1$  adrenoceptors. Thus, when the selective ratio of relative affinities of clonidine and phenylephrine is several times greater than unity,  $\alpha_2$  receptor is present, and *vice versa* (Wikberg, 1978).

### 2. Determination of Affinity or of Dissociation Constant of Antagonists – $pA_2$ or $pK_B$ Values

Antagonists are more reliable and more convincing tools than agonists in characterization or classification of receptors. Different tissues with similar receptors would be expected to give the same  $pA_2$  value with same agonist-antagonist pair (*see Chapter 19*). For example, with acetylcholine-atropine almost identical  $pA_2$  values are obtained in such varied preparations as frog heart, chick amnion and mammalian intestine, the one exception being the frog rectus that presumably has different (nicotinic) receptors (*see Table 19.3*).

### 3. Isomeric Activity Ratio of Agonists

Differences in potency between enantiomorphs (pairs of optical isomers) are determined on a particular tissue and the ratio obtained as described below.

*Isomeric activity ratio* = Antilog (negative log molar EC<sub>50</sub> of levoisomer minus negative log molar EC<sub>50</sub> of dextroisomer).

High ratio suggests a highly specific interaction with the receptor site. For instance, (-)-isoprenaline is about 35 times more potent than (+)-isoprenaline in producing positive chronotropic effect on guinea pig atria, while enantiomorphs of propranolol are equipotent in producing quinidine-like action on rabbit atria. Differences in potency between enantiomorphs may be due to differences in affinity alone, or in affinity and intrinsic activity (Bowman and Rand, 1980).

Since most of the pharmacological receptors are stereospecific, these can be classified into different types based on their ability to interact with optical isomers. The similarity of the ratios between enantiomorphs in different tissues indicates the similarity of the receptors with which the isomers interact. In order to get a reliable *isomeric activity ratio*, a number of factors such as neuronal and extraneuronal uptake, unequal distribution of antagonistically acting receptors in the same tissue, and the presence of degradation enzymes have to be controlled by the prior use of appropriate drugs.

Table 7.2 shows how diversified isomeric ratios (range 2–64) have been altered by drug pretreatment to give reasonably uniform ratios (range 50 – 80).

Table 7.2  
Isomeric activity ratio of optical isomers of noradrenaline (NA) on  
different tissues containing  $\alpha$ -adrenergic receptors\*

Test parameter	Pretreatment	Isomeric activity ratio (+)-NA vs. (-)-NA
Cat blood pressure	Normal	40
	Reserpine + cocaine	60
Cat nictitating membrane	Normal	8
	Reserpine + cocaine	80
Cat spleen	Normal	2
	Reserpine + cocaine	65
Rabbit aorta	Normal	42
	Normal	64
jejunum	Normal	64
	Reserpine + cocaine	5
Rat vas deferens	Normal	5
	Reserpine + desipramine	50

\* Adapted from Patil, 1969

### 4. Use of Radiolabelled Ligands

Radiolabelled ligands (agonist or antagonist) can also be used for isolation and characterization of receptors. For instance, labelled  $\alpha$ -bungarotoxin from snake venom binds specifically and irreversibly to cholinergic receptors of skeletal muscle. Labelled phenoxybenzamine, an irreversible  $\alpha_1$ -selective blocker has also been used for isolation and characterization of adrenoceptors.  $\beta$ -Adrenoceptors have been identified and characterized in many tissues with the help of highly potent labelled  $\beta$ -adrenergic blocking agents like ( $^3\text{H}$ )-dihydroalprenolol. The relative proportions of  $\beta_1$  and  $\beta_2$  receptors in a tissue can also be determined by ligand techniques. For example, in rat, the proportion of  $\beta_1$  and  $\beta_2$  receptors is about 4:1 in heart, 1:3 in lungs, and 2:1 in cerebral cortex (Barnett *et al.* 1978). The percentage of distribution between  $\beta_1$  and  $\beta_2$  adrenoceptors is about 20:80 in the guinea pig lung, and 30:70 in human lung (Engel, 1981).

### Experimental Conditions for the Pharmacological Characterization of Receptors\*

In order to draw a valid conclusion on the pharmacological characterization of receptors in isolated tissues, a set of optimal conditions has to be met as follows:

- a) The response to an agonist should be due solely to its direct action on only one type of receptor; in order to achieve this the unwanted receptors should be blocked with appropriate drugs,
- b) The response should not be due, even partially, to release of some active substance; this should be overcome by pretreatment of the animal with a suitable agent, for example, with reserpine,
- c) The concentration of free drug in the bathing solution should be maintained at a steady level with negligible rate of loss during the time a response is measured; this may be achieved by using neuronal and extra-neuronal uptake blockers, and metabolic enzyme (MAO, COMT) blockers (O'Donnell and Wanstall, 1976),
- d) The antagonist used should act competitively with the agonist for the receptor,
- e) The experimental design should include proper controls for corrections for any change in the tissue sensitivity to agonists during the course of the experiment, and
- f) Sufficient time has to be allowed for the antagonist to come into equilibrium with the receptors.

\* Furchgott, 1970.

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# Cholinergic Receptors

Dale classified cholinergic sites into two major types: *muscarinic* and *nicotinic* depending on the responsiveness of the receptors to these natural substances. The nicotinic receptors are now known to form an ion channel (ionotropic), and the muscarinic receptors to activate a variety of second messenger systems through GTP binding proteins i.e. G proteins (metabotropic). During the recent years, the subtypes of muscarinic as well as nicotinic receptors have been identified by molecular cloning.

## Muscarinic Acetylcholine Receptors (mAChRs)

They mediate various cellular responses including inhibition of adenylate cyclase, breakdown of phosphoinositides, and modulation of potassium channels through the action of guanine nucleotide binding regulatory proteins (G proteins). The cloning of cDNAs and genes for different mAChRs provides a new basis for characterizing muscarinic receptor function. Although as many as five types of muscarinic receptors have been detected, only three have been studied in detail. They are  $M_1$ ,  $M_2$  and  $M_3$  acetylcholine receptors. In recent years a number of drugs have been identified, notably pirenzepine that can discriminate between different subtypes of muscarinic receptors.

The distribution and characteristics of muscarinic receptors have been summarized in Table 8.1

Table 8.1  
Distribution and characteristics of different muscarinic acetylcholine receptors\*

	$M_1$	$M_2$	$M_3$
Location	Autonomic ganglia, Brain	Heart, Brain, Smooth muscle	Smooth muscle, Glands, Brain
Pirenzepine affinity	High	Low	Intermediate
PI <sup>o</sup> metabolism	+	0	+
cAMP response	+	-	+
Arachidonic acid response	+	0	+
Agonist	Oxotremorine McN-A343**	Nil	Nil
Antagonist	Pirenzepine** Atropine	Atropine Triptiramine**	Atropine Darifenacin**

\* Modified after Bonner, 1989

\*PI - Phosphatidylinositol  
- Inhibition

\*\* Selective  
0 No effect

+ Stimulation



## Nicotinic Acetylcholine Receptors (nAChRs)

They are classified on the basis of their activation by the alkaloid nicotine, the endogenous ligand being acetylcholine. Nicotinic acetylcholine receptors, known as ionotropic acetylcholine receptors, are a family of ligand-gated cation channels composed of five protein subunits. In mammalian brain, two major nAChR subclasses can be delineated using radioligand binding.

Table 8.2 summarizes the characteristics of different nicotinic acetylcholine receptors.

Table 8.2  
Distribution and characteristics of different nicotinic acetylcholine receptors

	$N_{\alpha}$	$N_{\beta}$	$N_{\gamma}$	$N_{\delta}$
Location	Neuromuscular junction	Autonomic ganglia, CNS Adrenal medulla	Mammalian brain	Mammalian brain
Radioligand binding with $\alpha$ -Bgt	-	-	High affinity ( $\alpha$ -Bgt sensitive)	No affinity ( $\alpha$ -Bgt insensitive)
Agonists	PTMA* Nicotine	DMPP* Nicotine Epibatidine*	Anatoxin DMAC	Cytisine, Anatoxin Epibatidine*
Antagonists	Tubocurarine $\alpha$ -Bgt	Trimethaphan	$\alpha$ -Bgt	Mecamylamine Dihydroerythroidin Erysidine

DMPP - Dimethylphenylpiperazinium  
PTMA - Phenyltrimethylammonium

DMAC - Dimethylacetamide  
 $\alpha$ -Bgt -  $\alpha$ -Bungarotoxin

\* Selective

Galantamine, a potent allosteric modulator, acts by binding to a site on nAChR that is different from the binding site of the natural agonist acetylcholine. This allosteric interaction amplifies the actions of acetylcholine at pre- and post-synaptic nAChRs.

Bupropion, an antidepressant agent, possesses some selectivity for nAChRs.

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# Adrenergic Receptors

Ahlquist in 1948 introduced the concept of two types of adrenergic receptors, (a) excitatory  $\alpha$  receptors, and (b) inhibitory  $\beta$  receptors. The  $\alpha$  receptors of intestine, however, are inhibitory and the  $\beta$  receptors of heart excitatory. In addition, many adrenoceptors in the central nervous system (CNS), and those mediating certain metabolic responses remain unclassified. Lands and coworkers in 1967, classified  $\beta$  receptors into two distinct types: (a)  $\beta_1$  receptors of cardiac and intestinal smooth muscles and adipose tissue, and (b)  $\beta_2$  receptors of bronchial, vascular and uterine muscles. Langer (1974) suggested the presence of two kinds of  $\alpha$  receptors: (a) postsynaptic  $\alpha_1$  adrenoceptors, and (b) presynaptic  $\alpha_2$  adrenoceptors, the latter subserving the function of a negative feedback mechanism. Bentley *et al.* (1977) further suggested that there might be two types of postsynaptic  $\alpha_1$  adrenoceptors: (a) prazosin-sensitive type stimulated by phenylephrine and blocked by prazosin, and (b) prazosin-insensitive type stimulated by low doses of noradrenaline but not blocked by prazosin. Phentolamine blocks, and high doses of noradrenaline stimulate both types of  $\alpha_1$  adrenoceptors. Receptors innervated by the right splanchnic nerve in cat seem to be predominantly prazosin-insensitive type that may resemble the presynaptic  $\alpha_2$  adrenoceptors located on the adrenergic terminals supplying the rabbit pulmonary artery and rat heart.

## Presynaptic or Prejunctional Receptors

Two types of presynaptic receptors are involved in the modulation of neurotransmitter release (Langer and Arbilla, 1981).

1. *Presynaptic inhibitory autoreceptors*, through which the neurotransmitter can regulate its own release, are present in the CNS for several neurotransmitters, such as noradrenaline, dopamine, acetylcholine, serotonin, gamma-aminobutyric acid (GABA) and possibly adrenaline. For noradrenaline, peripheral presynaptic autoreceptors are also in existence.
2. *Presynaptic receptors sensitive to other endogenous compounds different from the neuron's own transmitter such as:*
  - a) transmitters released from adjacent nerve terminals (axo-axonic synapses),
  - b) blood borne substances, like adrenaline or angiotensin II acting on presynaptic facilitatory receptors on noradrenergic nerve terminals, and
  - c) locally formed endogenous substances that may be involved in trans-

synaptic feedback mechanisms regulating transmitter release, as in the case of prostaglandins and adenosine modulating noradrenaline release.

Both the types of presynaptic receptors may be involved in the physiological control of transmitter release. They are also the target of drug action, either agonist or antagonist.

Two types of presynaptic autoreceptors are present on the surface of the noradrenergic nerve endings that are concerned in the regulation of noradrenaline release (Fig. 9.1).

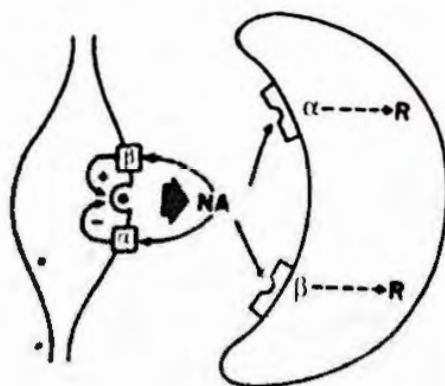


Fig. 9.1. Role of the presynaptic  $\alpha$  and  $\beta$  adrenoceptors in the regulation of noradrenaline (NA) release during nerve stimulation. During NA release at low frequencies of nerve stimulation (when the concentration of the released transmitter in the synaptic cleft is rather low) the positive feedback mechanism mediated by presynaptic  $\beta$  adrenoceptors is activated leading to an increase in transmitter release. As the concentration of released NA increases, a threshold is reached at which the negative feedback mechanism mediated by presynaptic  $\alpha$  adrenoceptor is triggered, leading to inhibition of transmitter release. Both presynaptic feedback mechanisms are present in nerves irrespective of the  $\alpha$  or  $\beta$  nature of the receptors that mediate the response (R) of the effector organ (Langer, 1977).

1. *Presynaptic  $\alpha_2$  adrenoceptors* – activated when higher concentration of noradrenaline is reached in the synaptic cleft leading to the inhibition of transmitter release, i.e. a *negative feedback mechanism*. This mechanism operates by restricting the calcium available for the excitation-secretion coupling.
2. *Presynaptic  $\beta$  adrenoceptors* – activated by low concentration of noradrenaline (i.e. in the range of low frequencies of nerve stimulation) that facilitates its further release, i.e. a *positive feedback mechanism*. This mechanism is mediated through an increase in the cyclic AMP levels in nerve endings.

Both the presynaptic feedback mechanisms are present in nerves irrespective of the  $\alpha$  or  $\beta$  nature of the receptors that mediate the response of the effector organ. The relative affinities of noradrenaline for the presynaptic  $\alpha_2$  and  $\beta$  adrenoceptors may be similar to that of postsynaptic adrenoceptors where 30 to 100 times higher concentration of noradrenaline are required to stimulate  $\alpha$  adrenoceptors than those necessary to stimulate the  $\beta$  receptors (Adler-Graschinsky and Langer, 1975).

The major regulatory mechanism for noradrenaline release by nerve stimulation under physiological conditions is mediated by presynaptic  $\alpha_2$  adrenoceptors, since the most pronounced increase in transmitter release is obtained when the presynaptic  $\alpha_2$  adrenoceptors are blocked by drugs. Presynaptic  $\alpha_2$  adrenoceptor modulation by synaptically released noradrenaline, however, plays no part in cardiac sympathetic transmission (Angus and Korner, 1980).

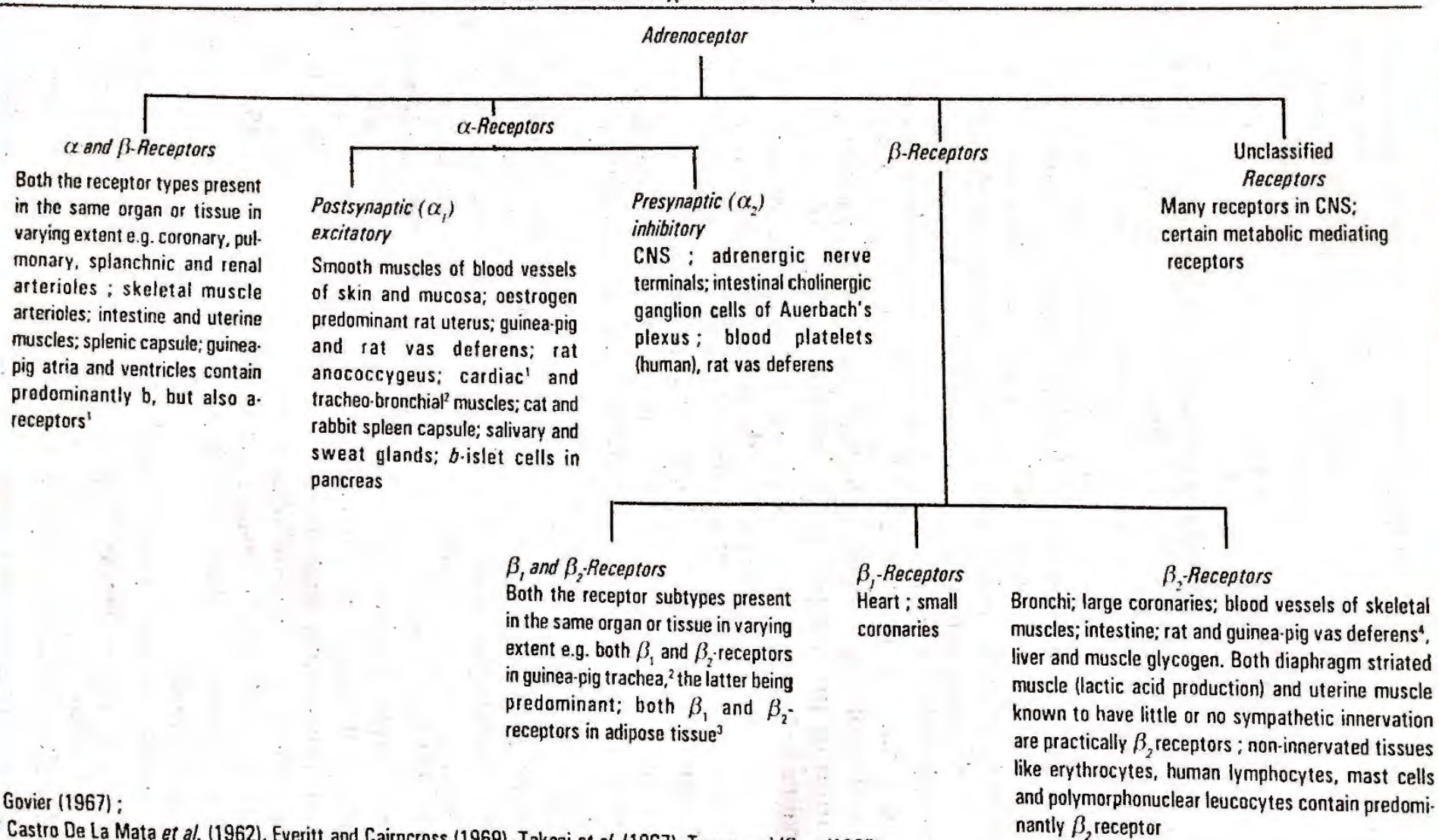
Differences exist between the presynaptic  $\alpha_2$  adrenoceptors and postsynaptic  $\alpha_1$  adrenoceptors as evidenced by the potency differences of agonists and antagonists at these two sites. For instance, agonists clonidine,  $\alpha$ -methylnoradrenaline and oxymetazoline are more potent in reducing noradrenaline release than in stimulating the postsynaptic  $\alpha_1$  adrenoceptors. The antagonist phenoxybenzamine is 30 to 100 times more potent in blocking the postsynaptic  $\alpha_1$  adrenoceptors than the presynaptic  $\alpha_2$  receptors. Though phenoxybenzamine in large concentration may enhance noradrenaline release, the same may not be evident on the effector organs, as the responses will be reduced by its predominant blocking action on the postsynaptic  $\alpha_1$  receptors. In a similar fashion, low concentration of isoprenaline enhances the release of noradrenaline through stimulation of presynaptic  $\beta$ -adrenoceptors. The effect obtained with (-)-isoprenaline is stereospecific, since it is not obtained with (+)-isoprenaline. Blockade of the presynaptic  $\beta$  adrenoceptors in the periphery contribute significantly to the antihypertensive effects of  $\beta$  receptor blockers. Yohimbine is more potent in blocking the presynaptic  $\alpha_2$  receptor than the postsynaptic  $\alpha_1$  receptor in the rabbit main pulmonary artery. Thus, low concentrations of yohimbine enhance the noradrenaline release, and also potentiate the responses to nerve stimulation without affecting the postsynaptic receptors.

In addition to the presynaptic  $\alpha_2$  and  $\beta$  adrenoceptors, a variety of presynaptic receptor sites have been described in noradrenergic nerve endings. These receptors are mostly inhibitory in nature, such as muscarine, dopamine, opiate, prostaglandin, 5-hydroxytryptamine and adenosine receptors, while angiotensin II and GABA are facilitatory receptors. Except for the  $\alpha_2$  and  $\beta$  presynaptic adrenoceptors, the other presynaptic receptors are not present in all the noradrenergic nerve endings. For example, the presynaptic opiate receptors are present in the rabbit and guinea pig heart; presynaptic angiotensin receptors are absent in the cat spleen, and prostaglandin E receptors are absent in the cat nictitating membrane. All these receptors, however, are of pharmacological importance, since they can be acted upon by agonists or their analogues to modify peripheral sympathetic neurotransmission.

*Presynaptic receptors in CNS.* A similar negative feedback mechanism by presynaptic  $\alpha_2$  adrenoceptors is operative in noradrenergic nerve endings in the brain. The antihypertensive effect of clonidine and other imidazolines may be through either presynaptic or postsynaptic  $\alpha$  adrenoceptors in the CNS or by both. Thus, the antihypertensive effect of clonidine is through activation of both

The distribution of different types of adrenoceptors in tissues is presented in Table 9.1.

**Table 9.1**  
Distribution of different types of adrenoceptors in tissues



<sup>1</sup> Govier (1967);

<sup>2</sup> Castro De La Mata *et al.* (1962), Everitt and Cairncross (1969), Takagi *et al.* (1967), Turner and Kiran (1965);

<sup>3</sup> Ablad *et al.* (1975). Befrage (1978);

<sup>4</sup> Ganguly and Bhattacharya (1970)

presynaptic  $\alpha_2$  adrenoceptors in the peripheral sympathetic system and  $\alpha$  adrenoceptors in the CNS (Langer, 1977).

Miach *et al.* (1978) have provided direct biochemical evidence of two types of  $\alpha$  adrenoceptor in rat brain.

## CLASSIFICATION OF ADRENOCEPTORS AND THEIR AGONISTS AND ANTAGONISTS

### A. $\alpha$ Adrenoceptors

The agonists and antagonists of  $\alpha$  adrenoceptors are presented in Table 9.2.

**Table 9.2**  
Agonists and antagonists of  $\alpha$ -adrenoceptors

Both postsynaptic $\alpha_1$ , and presynaptic $\alpha_2$ receptors		Postsynaptic $\alpha_1$ receptor (excitatory)		Presynaptic $\alpha_2$ receptor (inhibitory)	
Agonist	Antagonist	Agonist	Antagonist	Agonist	Antagonist
Adrenaline	Phenoxybenzamine	Phenylephrine	Prazosin*	Guanfacine*	Yohimbine*
Noradrenaline	Phentolamine	Methoxamine	Labetalol	Clonidine	
Naphazoline	Piperoxan	Isoprenaline	(+)Dobutamine	Tramazoline	
	Tolazoline	(-) Dobutamine	Indoramin*	Oxymetazoline*	
				$\alpha$ -Methylnor- adrenaline	

\* Selective

**Agonists.** Adrenaline, noradrenaline and naphazoline have agonistic activities on both post- and pre-synaptic  $\alpha$  receptors, adrenaline being more potent than noradrenaline. Adrenaline is equiactive or slightly more potent than noradrenaline on postsynaptic  $\alpha_1$  adrenoceptors (excitatory) present in smooth muscles of blood vessels, and in salivary and sweat glands. Phenylephrine, which is less potent than adrenaline, has almost pure  $\alpha_1$  activity. Isoprenaline has very weak activity on these receptors. Methoxamine, which does not cross the blood brain barrier, is devoid of any CNS effect; it has a low potency  $\beta$  antagonist activity (Patil *et al.* 1967). Clonidine, tramazoline, oxymetazoline and  $\alpha$ -methylnoradrenaline have agonistic action on presynaptic  $\alpha_2$  adrenoceptors (inhibitory) present in adrenergic and intestinal cholinergic nerve terminals, while guanfacine is a selective agonist. The (-) isomer of dobutamine is a potent  $\alpha_1$  receptor agonist, while its (+) isomer is a potent antagonist.

**Antagonists.** Prazosin has a selective, reversible and competitive blocking action on  $\alpha_1$  receptor. Yohimbine, a selective  $\alpha_2$  receptor antagonist, also blocks 5-HT receptor. Phenoxybenzamine is an insurmountable antagonist, while phentolamine is a surmountable antagonist, both being more potent at postsynaptic than at presynaptic receptors. Phenoxybenzamine in high doses also block histamine, 5-HT and acetylcholine receptors, as well as the neuronal and extraneuronal uptake of catecholamines. Phentolamine also blocks 5-HT receptors, and produces histamine release from mast cells. Tolazoline stimulates exocrine glands. Indoramin also blocks the  $H_1$  and 5-HT receptors.

Tamsulosin is a subtype-selective ( $\alpha_{1A}$  &  $\alpha_{1D}$ ) adrenoceptor antagonist. Alpha<sub>1</sub>-receptors predominate in the prostate gland, capsule, and urethra as well as in bladder. By binding to these receptors, tamsulosin relaxes smooth muscles in the prostate and bladder neck thereby increasing the urine flow and reducing the symptoms of benign prostatic hyperplasia (BPH). Compared to prazosin and terazosin, tamsulosin is more selective for the  $\alpha_1$ -receptors.

## B. $\beta$ -Adrenoceptors

The agonists and antagonists of  $\beta$  adrenoceptors are presented in Table 9.3

**Table 9.3**  
Agonist and Antagonist of  $\beta$ -adrenoceptors

Both $\beta_1$ (cardiac) and $\beta_2$ Receptors		$\beta_1$ Receptor (cardiac) (excitatory)		$\beta_2$ Receptor (extracardiac) (inhibitory)	
Agonist	Antagonist	Agonist	Antagonist	Agonist	Antagonist
Isoprenaline	Propranolol	Dopamine	Practolol	Methoxyphenamine	Butoxamine
Adrenaline	Oxprenolol	(+) Dobutamine	Acebutolol	Salbutamol	H 35/25
Noradrenaline	Pindolol	Isoprenaline	Atenolol	Terbutaline	
	Sotalol		Metoprolol	Orciprenaline	
	Timolol		Tolamolol	Isoetharine	
	Nadolol		Celiprolol	Celiprolol	
	Alprenolol			Ritodrine	
	Labetalol				

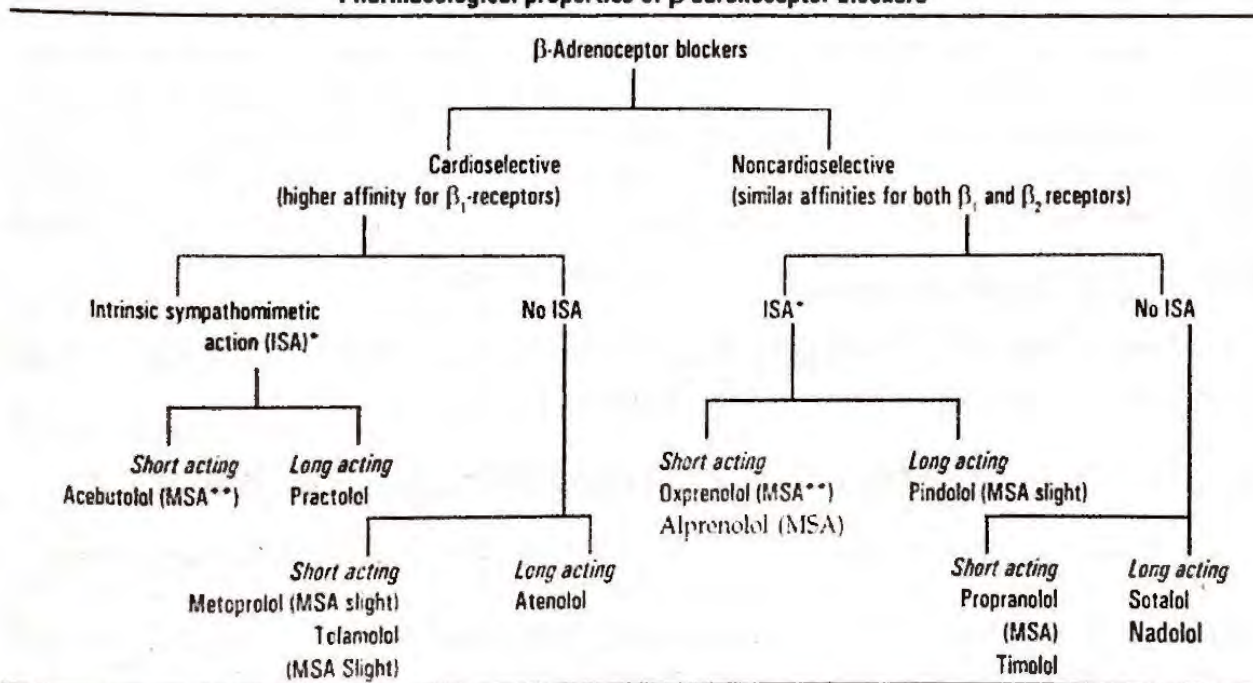
**Agonists.** Isoprenaline is about 10 times more effective on  $\beta_1$  receptors than adrenaline and noradrenaline, which are equiactive, while it has similar free fatty acid (FFA) releasing activity as adrenaline. It also stimulates insulin secretion by  $\alpha$ -adrenergic activation of pancreatic islet cells. As a bronchodilator, isoprenaline is about four times more potent than adrenaline, whilst noradrenaline is 10 times less potent than adrenaline. Adrenaline has more hyperglycaemic effect than isoprenaline. Dobutamine is a  $\beta_1$  receptor agonist, its (+) isomer being 10 times more potent than the (-) isomer. Dopamine has a positive inotropic effect on

myocardium. Salbutamol is 10 times more selective on  $\beta_2$ -receptors (bronchial) than on  $\beta_1$ -receptors (cardiac). Isoprenaline and salbutamol though equipotent, the former also produces cardiac stimulation being equieffective on both  $\beta_1$  and  $\beta_2$  receptors. Besides bronchial relaxation, the  $\beta_2$  agonists may suppress the release of leukotrienes and histamine from mast cells in the lung tissue (Hughes *et al.* 1983). Ritodrine, a  $\beta_2$  agonist has a specific uterine relaxant property.

**Antagonists.** Timolol and pindolol are five to ten times more potent than propranolol, while nadolol and sotalol are less potent. Pindolol, oxprenolol and acebutolol also act as partial agonists. Practolol and metoprolol are 10 to 20-fold cardioselective, while atenolol is 3-fold cardioselective. Celiprolol, a  $\beta_1$  antagonist has a mild  $\beta_2$  agonist activity. Both butoxamine and H 35/25 block isoprenaline-induced vasodilatation. The latter has also other smooth muscle inhibitory effect, and blocks increase in glucose, lactate and plasma FFA.

The pharmacological properties of  $\beta$ -adrenoceptor blockers are presented in Table 9.4.

**Table 9.4**  
Pharmacological properties of  $\beta$ -adrenoceptor blockers<sup>†</sup>



<sup>†</sup> Opie (1980), Wilcox (1978)

\* Partial agonist

\*\* Membrane stabilizing, local anaesthetic and quinidine-like action

### C. Both $\alpha$ and $\beta$ Adrenoceptors

**Agonists.** The effect of noradrenaline is more marked on  $\alpha$  and  $\beta_1$  receptors. Many non-catecholamines like ephedrine that releases noradrenaline, have predominantly  $\alpha$  and  $\beta_1$  (cardiac) effects. Ephedrine has in addition a direct  $\beta_2$



effect producing bronchodilatation. Isoprenaline elicits responses at  $\beta$  receptors in nanomolar concentration, while it activates  $\alpha$  receptors in much higher (micromolar) concentration (Jenkinson, 1973).

*Antagonists.* Labetalol possesses a unique combination of both  $\alpha$  and  $\beta$  adrenoceptor blocking activity, being four to eight times more potent against  $\beta$  than against  $\alpha$  adrenoceptors. It is one and a half to three times less potent than propranolol in blocking  $\beta$  adrenoceptors, and six to ten times less potent than phentolamine in blocking  $\alpha$  adrenoceptors. Labetalol does not possess any intrinsic sympathomimetic activity. However, it appears to possess  $\beta_2$  agonist activity on the isolated spontaneously contracting rat uterus (Carey and Whalley, 1979). It also inhibits reuptake of noradrenaline into nerve terminals.

Carvedilol is a nonselective  $\beta$ -adrenergic ( $\beta_1$  &  $\beta_2$ ) as well as  $\alpha_1$  adrenergic blocker. It is a potent antihypertensive agent with a dual mechanism of actions. At relatively low concentration it is a competitive  $\beta$ -adrenoceptor antagonist and a vasodilator, whereas at higher concentration it is also a calcium channel antagonist. Carvedilol is also a potent  $\alpha_1$ -adrenoceptor antagonist which accounts for most, if not all, of the vasodilating response produced by the compound. The vasodilating activity of carvedilol results largely from  $\alpha_1$  adrenoceptor blockade, while its  $\beta$ -adrenoceptor blocking activity prevents reflex tachycardia. Relative to other  $\beta$ -blockers carvedilol has minimal inverse agonist activity. This suggests carvedilol has a reduced negative chronotropic and inotropic effect compared to the other  $\beta$  blockers. Carvedilol is indicated in hypertension, heart failure caused by ischemic cardiomyopathy and left ventricular dysfunction following myocardial infarction.

#### D. Unclassified Adrenoceptors

Many adrenoceptors in CNS, and those mediating certain metabolic functions have not yet been identified precisely (Table 9.1).

#### NATURE OF CARDIAC ADRENOCEPTORS

The cardiac adrenoceptors are entirely of the  $\beta$  type belonging mainly to the  $\beta_1$  subgroup. However,  $\beta_2$  adrenoceptors are also present to a varying extent in the heart of different species. For instance,  $\beta_2$  adrenoceptors are present in small proportion in guinea pig and dog hearts, and in large proportion in cat and human hearts. It is possible that at least in some species,  $\beta_2$  adrenoceptors are mainly involved in the mediation of positive inotropic effect, whereas  $\beta_1$  adrenoceptors are primarily concerned in mediating positive chronotropic effect.

The existence of myocardial  $\alpha$  adrenoceptors has been demonstrated in rat ventricle and in rabbit and guinea pig atria.  $\alpha$ -Adrenoceptors do not respond to phenylephrine when the heart is under control of the normal pacemaker. However, in the driven rabbit left atrium, phenylephrine produces positive inotropic effect through  $\alpha$ -adrenoceptor stimulation, which can be blocked by  $\alpha$  adrenoceptor antagonists, like phentolamine and phenoxybenzamine (Benfey, 1973).

**TISSUES OR ORGANS EMPLOYED FOR THE STUDY OF DIFFERENT ADRENOCEPTOR SYSTEMS**

**Postsynaptic  $\alpha_1$ -Receptor System**

- Rat isolated vas deferens (contraction)
- Rat isolated anococcygeus muscle (contraction)
- Rabbit and guinea pig isolated aorta (contraction)
- Rabbit isolated jejunum (inhibition).
- Guinea pig isolated ileum (inhibition).
- Cat nictitating membrane (contraction)

**Presynaptic  $\alpha_2$ -Receptor System**

- Transmurally stimulated rat isolated vas deferens at low frequency (inhibition).
- Electrically stimulated guinea pig isolated ileum, and rabbit isolated ear artery, pulmonary artery and jejunum (inhibition).

**$\beta_1$ -Receptor System**

- Rabbit isolated perfused heart (contraction)
- Rabbit isolated jejunum (inhibition)
- Minced testicular adipose tissue of rat (lipolysis)

**$\beta_2$ -Receptor System**

- Guinea pig perfused lung (bronchodilatation)
- Guinea pig isolated trachea (inhibition)
- Rat isolated uterus (inhibition)
- Anaesthetized dog blood pressure (depression)

**Both  $\alpha$  and  $\beta$ -Receptor Systems**

Mouse isolated spleen ( $\alpha$  – contraction,  $\beta$  – relaxation) (Ignarro and Titus, 1968).

For localization of presynaptic effects of drugs see Table 9.5

**Table 9.5**  
**Localization of presynaptic effect of drugs**

<i>Rat isolated vas deferens*</i>	<i>Guanethidine</i> 3 $\mu\text{g/ml}$ .	<i>Bretylium</i> 10 $\mu\text{g/ml}$ .	<i>Clonidine</i> 0.1-3 $\mu\text{g/ml}$ .	<i>Papaverine</i> 100 $\mu\text{g/ml}$ .	<i>Mechanism</i>
Low frequency field stimulation (0.1 Hz, 3 ms, 10-30 v)	-	-	-	-	Inhibition through neuron blocking, presynaptic and direct effects
Direct muscle stimulation (0.1 Hz, 100 ms, 100v) or nor-adrenaline evoked	0	0	0	-	Inhibition by direct effect

Table 9.5 (Contd.)

<i>Rat isolated vas deferens*</i>	<i>Guanethidine</i> 3 µg/ml.	<i>Bretylum</i> 10 µg/ml.	<i>Clonidine</i> 0.1-3 µg/ml.	<i>Papaverine</i> 100 µg/ml.	<i>Mechanism</i>
Low frequency field stimulation of the preparation pretreated with yohimbine and phentolamine	-	-	0		Presynaptic inhibition by clonidine, antagonized by α <sub>2</sub> -adrenoceptor blockers

\* The tissue is bathed in Kreb's solution containing corticosterone 40 µM, desipramine 10 µM and propranolol 0.1 µM to exclude the influence of extraneuronal and neuronal uptake of catecholamines, and of the β-effects.

- inhibition; 0 no effect

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# Dopaminergic, Serotonergic and Gaba Receptors

## DOPAMINERGIC RECEPTORS

The cloned dopamine (DA) receptors are divided into two classes: (i)  $D_1$ -like pharmacological profile receptors ( $D_1$  and  $D_5$ ), and (ii)  $D_2$ -like pharmacological profile receptors ( $D_2$ ,  $D_3$  and  $D_4$ ). In general the  $D_1$  and  $D_2$  receptor mRNAs have a wider distribution and are more abundant in the CNS. Both the receptor classes are present in the nigrostriatal and mesocorticolimbic pathways in CNS. They exert their biological actions by coupling to and activating different G protein complexes.

The properties of DA receptors are summarized in Table 10.1

Table 10.1  
Properties of dopaminergic receptors

Criteria	$D_1$ -like Receptors	$D_2$ -like Receptors
Distribution	CNS, Stomach, Intestine	CNS, Anterior pituitary, Stomach, Intestine
Interaction with G-protein complexes	$G_s$ complex - stimulation of adenylylase	$G_i$ complex - inhibition of adenylylase
Cyclic AMP	Increased	Decreased
Gastric motility, mucosal blood flow, acid secretion	Inhibited	Stimulated
Gastric and duodenal ulcers	Anti-ulcer effect	Pro-ulcerogenic effect
Dopamine (agonist)	Low affinity ( $\mu\text{M}$ conc.)	High affinity (nM conc.)
Apomorphine	Partial agonist / antagonist	Agonist (nM conc.)
Bromocriptine and Lisuride	Weak agonist ( $\mu\text{M}$ conc.)	Potent agonist (nM conc.)
Agonists	SKF-38393*, Fenoldopam* Piribedil, Pergolide Bromocriptine (partial)	Bromocriptine*, N-0437* Pergolide, Lisuride, Pramipexole*, Ropinirole*
Antagonists	SCH-23390*, SCH-39166*, Pimozide*, Spiperone Clozapine (high affinity) Butaclamol Bromocriptine*	Sulpiride*, Domperidone* Metoclopramide, Spiperone, Haloperidol, Risperidone Clozapine (low affinity) Cisapride

\* Selective agonist / antagonist

SEROTONERGIC (5-HYDROXYTRYPTAMINE) RECEPTORS

Gaddum and Picarelli (1957) suggested that 5-hydroxytryptamine (5-HT) or serotonin interacted with two different receptors in isolated tissues; (i) smooth muscle, the contraction of which is blocked by dibenzyline, hence named D receptors, and (ii) parasympathetic nerve endings controlling the release of acetylcholine, named M receptors, as morphine was selective on these tissues.

With the availability of newer techniques, especially with cloning studies, it appears that there are at least seven types of serotonin receptors of which only four types (5-HT<sub>1</sub> – 5-HT<sub>4</sub>) with their subtypes have been studied in detail. The 5-HT<sub>1</sub> receptors are further classified into A, B, and D subtypes, and 5-HT<sub>2</sub> receptors into A, B, and C subtypes. All the 5-HT receptors belong to the G protein-coupled receptors (GPCRs) except 5-HT<sub>3</sub> receptor, which is a ligand-gated cation channel that corresponds to M receptor. Linked to the inhibitory G proteins, 5-HT<sub>1</sub> has an inhibitory effect on adenylyl cyclase, and on neurotransmission when bound by an agonist. Linked to stimulatory G proteins, 5-HT<sub>2</sub> receptors through activation of phospholipase C, and 5-HT<sub>4</sub> receptors through activation of adenylyl cyclase have a stimulatory effect on neurotransmission when bound by an agonist.

The properties of 5-HT receptors are summarized in Table 10.2

Table 10.2  
Properties of 5-HT receptors

	5-HT <sub>1</sub>	5-HT <sub>2</sub>	5-HT <sub>3</sub>	5-HT <sub>4</sub>
Location	CNS Cerebral blood vessels	CNS PNS Platelets (A) Vascular smooth muscles, Stomach fundus	CNS PNS GI tract	Brain GI tract Heart Bladder
Family	GPCR	GPCR	Ligand-gated cation channel	GPCR
Neurotransmission	Inhibition by linking to G <sub>i</sub> protein	Stimulation by linking to G <sub>s</sub> protein		Stimulation by linking to G <sub>s</sub> protein
Agonist	Sumatriptan (D) Buspirone (A)**	LSD (A & C) in CNS	LSD 2-Me-5-HT	Cisapride <sup>a</sup>
Metoclopramide <sup>a</sup>		Zolmitriptan (B & D)		LSD
Antagonist	Naratriptan (B & D) Ergotamine; LSD (A) Flesinoxan (A)**	Methysergide (A&C) Ketanserin (A & C) LSD (A) in periphery Cyproheptadine (A) on smooth muscles	Ondansetron Granisetron Tropisetron Alosetron	Ondansetron (weak)

Table 10.2 (contd.)

5-HT <sub>1</sub>	5-HT <sub>2</sub>	5-HT <sub>3</sub>	5-HT <sub>4</sub>
	Metergoline, Trazodone Clozapine (A & C)* Risperidone (A)* Ritanserlin (A & C)		

( ) subtype of 5-HT receptors

\* Also antidopaminergic (D<sub>2</sub>) and antihistaminic (H<sub>1</sub>)

• High affinity for  $\alpha$ -adrenergic and H<sub>1</sub> receptors

PNS Peripheral nervous system

\*\* Partial agonist

▲ Also antidopaminergic (D<sub>2</sub>)

### Serotonin Reuptake Inhibitors

Clomipramine, a potent inhibitor of 5-HT uptake also inhibits noradrenaline uptake, has affinity for central dopamine D<sub>2</sub>, histamine H<sub>1</sub> and  $\alpha_1$ -adrenergic receptors, and possesses anticholinergic effect. Tertiary antidepressants (TCAs), specially, the tertiary amines imipramine and amitriptyline are more selective uptake blockers of 5-HT than of catecholamines. Venlafaxine is a potent inhibitor of both 5-HT and NA uptake and weak inhibitor of DA uptake.

*Selective serotonin reuptake inhibitors (SSRI)*. Paroxetine most potent, sertraline less potent, and fluoxetine least potent selective inhibitors. Sertraline is the most selective for 5-HT versus NA uptake, while fluoxetine less selective. Fluvoxamine is a potent and selective 5-HT uptake inhibitor. Trazodone, a selective 5-HT reuptake blocker, also acts as an antagonist of 5-HT and presynaptic  $\alpha_2$ -NA receptors. It is metabolized to m-chlorophenylpiperazine, a known agonist at 5-HT receptor and an inhibitor of 5-HT uptake.

### Serotonin depletors

Fenfluramine, a selective long acting agent, and MDMA (Methylenedioxyamphetamine) are serotonin depletors.

## GABA RECEPTORS

GABA or  $\gamma$ -aminobutyric acid is the main inhibitory neurotransmitter in the central nervous system. Glutamic acid decarboxylase (GAD) catalyses the formation of GABA from L-glutamate. Three distinct types of GABA receptors have been characterized as follows:

*GABA<sub>A</sub> receptor* – prevalent in the mammalian brain, is a ligand-gated Cl<sup>-</sup> ion channel that is opened after release of GABA from presynaptic neurons. It is responsible for the inhibitory neurotransmission in the CNS. This receptor has three basic binding sites, ( i ) GABA site ( *b* subunit), (ii) benzodiazepine site ( *a* subunit), and (iii) barbiturate site. GABA binds to *b* subunit. Benzodiazepines and barbiturates bind to *a* subunit and to barbiturate site respectively, enhancing the GABA-induced ionic currents through these channels, that is, they modulate the effects of GABA.

*GABA<sub>B</sub> receptor* – present in high concentration in the interpeduncular nuclei and cerebellum, is a G protein coupled receptor (GPCR) coupled both to biochemical pathways and to regulation of ion channels, a class of receptor referred to as metabotropic.

*GABA<sub>C</sub> receptor* – widely distributed in the CNS, and most prominently in retinal neurons, belongs to ligand-gated ion channel family.

**GABA Reuptake Inhibitor**

Nipecotic acid, 2-Hydroxy GABA, Guvacine (specific)

**Inhibitor of GABA Degradation**

Amino-oxyacetic acid

**GABAergic Neurotoxin**

Kainic acid

The properties of different types of GABA receptors are summarized in Table 10.3

**Table 10.3**  
Properties of GABA receptors

Criteria	GABA <sub>A</sub>	GABA <sub>B</sub>	GABA <sub>C</sub>
Distribution	Mammalian brain	Cerebellum Interpeduncular nuclei	Retinal neurons CNS
Category	LGCC	GPCR	LGCC
Agonist	GABA, Muscimol (potent), Isoguvacine <sup>Δ</sup> , THIP	GABA, (R)-Baclofen, SKF 97541	GABA, Muscimol* CACA*, Isoguvacine*
Modulator	Diazepam, Barbiturates Lorazepam (α subunit)		Zinc
Antagonist	Bicuculline (potent) Flumazenil, Hydrastine SR 95531 <sup>Δ</sup> , Picrotoxin	(R)-Phaclofen SCH 50911, Saclofen, CGP 52432	TPMPA <sup>Δ</sup> , Picrotoxin, Low conc. of zinc ion THIP

GPCR – G protein coupled receptor

LGCC – Ligand-gated chloride channel

<sup>Δ</sup>Selective

CACA – cis-amino crotonic acid

THIP – Tetrahydro isooxazole pyridinol

\* Partial agonist

TPMPA – (1,2,5,6-Tetrahydropyridin-4-yl) methylphosphonic acid

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# Histaminergic Receptors

The existence of two types of histamine receptors,  $H_1$  and  $H_2$  is well known. A third type of histamine receptor  $H_3$  has been described by Arrang *et al.* (1983) as a presynaptic receptor present in histamine containing nerve terminals. It regulates the release and synthesis of histamine through an auto-inhibitory feedback mechanism. It also regulates the release of acetylcholine, noradrenaline, dopamine and serotonin (Leurs *et al.* 1998). All the three H receptors have recently been cloned and  $H_1$  and  $H_2$  receptors shown to be G protein-coupled receptors.  $H_1$  receptors are coupled to phospholipase C leading to a chain of reactions ultimately producing a response in the target cell.  $H_2$  receptors are linked to the stimulation of adenylyl cyclase resulting in the activation of cAMP-dependent protein kinase in the target cell.  $H_3$  receptor occupation, on the other hand, leads to decreased  $Ca^{2+}$  influx into the cell. The histamine homologues discriminate between the  $H_3$  receptors in the cerebral cortex and in the intestinal strip preparations of guinea pig and mouse suggesting that there could be two subtypes of  $H_3$  receptors (Leurs *et al.* 1996; Harper *et al.* 1999).

## Distribution of H-Receptors

In mammalian brain  $H_1$  receptor uses  $Ca^{2+}$ , and  $H_2$  receptor uses cAMP as second messengers. Some tissues contain either  $H_1$  or  $H_2$  receptors, while others contain both  $H_1$  and  $H_2$ -receptors. The  $H_3$  receptors have been demonstrated in CNS, gastrointestinal tract and cardiovascular systems.

The distribution of histamine receptors in different tissues are presented in Table 11.1

Table 11.1  
Distribution of histamine receptors in different tissues

Preparation	$H_1$ receptor	$H_2$ receptor	Effect
Guinea pig			
ileum	+	+	( $H_1$ ) contraction ( $H_2$ ) relaxation*
uterus	+	+	( $H_1$ ) ( $H_2$ ) contraction
tracheobronchial muscle**	+++	+	( $H_1$ ) contraction ( $H_2$ ) relaxation
pulmonary vascular bed	+	+	( $H_1$ ) pressor ( $H_2$ ) depressor
Cat trachea	+	+	Relaxation
Human bronchus	++	+	( $H_1$ ) contraction ( $H_2$ ) relaxation

# Histaminergic Receptors

Table 11.1 (Cont.)

Preparation	H <sub>1</sub> receptor	H <sub>2</sub> receptor	Effect
Rat anaesthetized stomach	+	0	Contraction
gastric secretion	0	+	Stimulation
isolated uterus	0	+	Relaxation through release of NA
Rabbit aorta and ear artery	+	+	(H <sub>1</sub> ) vasoconstriction
		(H <sub>2</sub> ) vasodilatation	
Blood pressure dog, cat, rat, rabbit	+	+	Depressor
guinea pig	+	0	(H <sub>1</sub> ) pressor (H <sub>2</sub> ) depressor
			Depressor

+ presence      0 absence      \* Muley *et al.* 1984      \*\* Okpako *et al.* 1978

**Cardiac histamine receptors.** The distribution of histamine receptors in the heart differs from species to species, and also within the heart in the same species as shown in Table 11.2. The cardiac H<sub>2</sub>-receptors, but not H<sub>1</sub>-receptors, are associated with adenylate cyclase-cyclic AMP system.

Table 11.2  
Distribution of histamine receptors in the heart of different species

Species	H <sub>1</sub> -receptor	H <sub>2</sub> -receptor	Effect
Rat	0	0	Nil
Cat	0	+	Contraction
Avian, rabbit, dog	+	0	Contraction
Guinea-pig			
left atrium	+	0	+ve inotropic
right artium	0	+	+ve chronotropic
left ventricle	+	+	(H <sub>1</sub> )-ve inotropic (H <sub>2</sub> )+ve inotropic
right ventricle	0	+	+ve inotropic
Human heart and coronary artery <sup>1</sup>	+	+	(H <sub>1</sub> ) contraction of coronary artery smooth muscle; no effect on myocardium; (H <sub>2</sub> ) slight relaxation of coronary artery smooth muscle; +ve inotropic action on myocardium

<sup>1</sup> Ginsburg *et al.*, 1980

**Histamine receptors in blood vessels.** Histamine by acting on both H<sub>1</sub> and H<sub>2</sub> receptors relaxes smooth muscle of terminal arterioles thereby passively producing capillary dilatation. It also increases capillary permeability, partly by separation of the endothelial cells by the passive dilatation of the post-capillary venules, and

partly by direct contraction or shrinkage of the endothelial cells acting through  $H_1$  receptors.

In rabbit, large doses of histamine produce a biphasic response, initially a rise mediated by  $H_1$  receptors, followed by a fall mediated through  $H_2$  receptors.

There is an increasing evidence that histamine may be a neurotransmitter in the mammalian CNS. The  $H_2$  receptor in the brain is coupled to adenylate cyclase, the activation of which by histamine may be an early step in the sequence of biochemical events through which certain physiological effects in the CNS are exerted. A large number of drugs of chemically diverse nature having antidepressant property are also potent inhibitors of histamine-sensitive adenylate cyclase (Kanof and Greengard, 1978).

### Identification of H Receptors

Differentiation of the three receptor types can be made by using agonists and antagonists that are relatively specific for each receptor (Table 11.3). For identification of  $H_1$  receptors, contractions of guinea pig isolated ileum and of anaesthetized rat stomach have been used, while for identification of  $H_2$  receptors, rate of contraction of guinea pig isolated atria, inhibition of electrically contracted uterus of rat, and rat gastric acid secretion have been employed (Black *et al.* 1972). For identification of  $H_3$  receptors, intestinal-strip preparations of guinea pig and mouse have been used (Leurs *et al.* 1996; Harper *et al.* 1999).

Table 11.3  
Agonist and antagonist of different H receptors

Receptor	Agonist	Antagonist
$H_1$	Histamine 2-Methylhistamine 2-Pyridylethylamine 2-Thiazolyethylamine	Diphenhydramine Chlorpheniramine Promethazine Loratadine
$H_2$	Histamine 4-Methylhistamine Dimaprit Impromidine	Cimetidine Ranitidine Famotidine Burimamide
$H_3$	(R)- $\alpha$ -Methylhistamine* Imetit Immepip	Thioperamide* Clobenpropit Burimamide Impromidine

\* Selective

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# Neuronal Uptake Blockers and Biogenic Amine Depletors

The main physiological importance of neuronal uptake is transmitter economy. The transmitter noradrenaline (NA) after being released following nerve stimulation is taken back mostly into the nerve terminals and retained for future use. The primary mechanism of the termination of actions of endogenous and exogenous catecholamines is by uptake into the sympathetic nerve terminals (neuronal uptake). A very minor role is played by the uptake by other mechanisms (extraneuronal uptake), viz., uptake by extraneuronal cells, dilution by diffusion (overflow), metabolic transformation by monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT). Extraneuronal uptake mechanism becomes important when the neuronal uptake is blocked or over saturated, or a very large concentration of catecholamines are used.

The differences between the two uptake mechanisms, neuronal and extraneuronal are presented in Table 12.1

**Table 12.1**  
Differences between the two uptake mechanisms\*

<i>Criteria</i>	<i>Neuronal (Uptake,<sub>1</sub>)</i>	<i>Extraneuronal (Uptake,<sub>2</sub>)</i>
Tissues involved	Central and peripheral sympathetic neurons	Post-synaptic cells, such as smooth muscles, gland cells, myocardium
Membrane carrier-system (Na <sup>+</sup> dependent) requiring metabolic energy	Dependent	Less dependent
Uptake process	Saturable	Saturable
Affinity for adrenaline (A) and noradrenaline (NA)	NA >> A	Affinity for both low A > NA
Capacity	Low	High
Stereoselectivity in rat tissues	Stereoselective (-)-NA > (+)-NA	Not stereoselective
Substrates	NA, Tyramine, Octopamine Metaraminol, $\alpha$ -Methyl-adrenaline $\alpha$ -Methyl-noradrenaline $\alpha$ -Methyl-tyramine	Isoprenaline

## Neuronal Uptake Blockers and Biogenic Amine Depletors

Table 12.1(Contd.)

Criteria	Neuronal (Uptake <sub>1</sub> )	Extraneuronal (Uptake <sub>2</sub> )
Uptake and re-use	++	Uptake followed by degradation by MAO and COMT
Facilitation of uptake process	Lithium, increase in extracellular Na <sup>+</sup> concentration	No such action
Inhibition of uptake	Desipramine, Metaraminol (+)-Amphetamine, Cocaine, PBA, Propranolol	SKF 550, SKF 625A 17- $\beta$ -Oestradiol, PBA in high conc. Corticosterone Propranolol inhibits NA uptake into platelets

\* Iversen, 1973

PBA - Phenoxybenzamine

In higher concentration, desipramine and cocaine also block the effects of sympathetic nerve stimulation by virtue of their local anaesthetic action; desipramine in very high concentration produces blocking of muscarinic and serotonergic receptors and of serotonin uptake. PBA has inhibitory action on presynaptic  $\alpha_2$  adrenoceptors. Metaraminol and tyramine being uptake<sub>1</sub> substrates enter the sympathetic nerve. Hence, their action is generally antagonized rather than potentiated by uptake<sub>1</sub> inhibitors, such as cocaine. Bretilium and guanethidine are selectively concentrated by uptake<sub>1</sub> process in adrenergic nerve terminals thereby causing neuronal block. 6-Hydroxydopamine is selectively accumulated in adrenergic nerves by uptake<sub>1</sub> mechanism thereby producing chemical lesion leading to the selective destruction of both peripheral and central adrenergic neurons.

### DOPAMINE

In the CNS, dopamine (DA) containing neurons possess their own specialized uptake system for DA. The DA uptake process which is sodium-dependent has the following characteristics: (i) a very high affinity for DA and a low affinity for NA, (ii) does not show any stereoselectivity for the isomers of NA, (iii) inhibited by both stereoisomers of amphetamine, (+)-isomer being slightly more potent than (-)-isomer, (iv) very slightly blocked by desipramine, and (v) markedly inhibited by benztropine, an antiparkinsonian drug.

### 5-HYDROXYTRYPTAMINE

5-Hydroxytryptamine (5-HT) is taken up by a specific sodium-dependent high affinity mechanism in tryptaminergic neurons in the brain. It is also taken up, though to a very less extent by both noradrenergic and dopaminergic neuronal uptake sites. 5-HT uptake is inhibited by various indolamines and tertiary amine

derivatives of imipramine and amitriptyline, 3-chloroimipramine being the most potent inhibitor. There is a close parallelism between the uptake mechanisms in platelets and in tryptaminergic neurons, both being inhibited by tricyclic antidepressants (Gordon and Overman, 1976). 5,6-Dihydroxytryptamine has a selective neurotoxic action on 5-HT containing neurons by virtue of its selective concentration in these nerves compared to NA or DA uptake sites.

### CHOLINE

The high affinity uptake of choline by cholinergic nerves is an important mechanism for recapturing this essential transmitter precursor substance after acetylcholine has been released from cholinergic nerve terminals and enzymatically hydrolyzed in the synaptic cleft. This may be particularly important for cholinergic neurons in the CNS, since brain tissue is incapable of synthesizing choline *de novo*. Hemicholinium-3 is a potent inhibitor of choline uptake by the cholinergic neurons. It competes with choline for the carrier molecules, thus preventing the passage of choline into the axoplasm.

Selective blockers of neuronal uptake mechanism are presented in Table 12.2

Table 12.2  
Neuronal uptake blockers

Type of Neuron	Blocker
Noradrenergic (NA)	Desipramine, Metaraminol Venlafaxine, (+)-Amphetamine, Cocaine, Phenoxybenzamine, Propranolol, Clomipramine
Dopaminergic (DA)	Amantadine, Benztropine Amitriptyline, HA-966
Serotonergic (5-HT)	Paroxetine***, Sertraline** Venlafaxine, Fluoxetine*, Imipramine, Amitriptyline, Clomipramine <sup>†</sup> , Desipramine (high conc.), Trazadone, Fluvoxamine***
GABAergic (GABA)	Nipecotic acid, 2-Hydroxy-GABA
Purinergic	Dipyridamole, Hexabendine
Cholinergic (ACh)	Hemicholinium-3 bromide (HC-3)

\*\*\* Most potent

\*\* Intermediate potency

\* Least potent; these are all selective serotonergic receptor inhibitors (SSRIs)

<sup>†</sup> Also inhibits NE uptake; has affinity for dopamine D<sub>2</sub> (central), histamine H<sub>1</sub> and adrenergic α<sub>1</sub> receptors. Sertraline is the most selective blocker for 5-HT versus NE uptake.

Different biogenic amines and their depletors along with their mechanism of action are presented in Table 12.3.

## Neuronal Uptake Blockers and Biogenic Amine Depletors

Table 12.3  
Biogenic amine depletors

<i>Biogenic amine</i>	<i>Depletor</i>	<i>Acts by inhibition of</i>
Noradrenaline & dopamine	$\alpha$ -Methyl-p-tyrosine ( $\alpha$ -MPT)	Rate limiting enzyme tyrosine hydroxylase
	$\alpha$ -Methyldopa, L- $\alpha$ -methyl-dopa hydrazine (carbidopa)* Benserazide*	Dopa decarboxylase
	Reserpine, Tetrabenazine**	Granular uptake and storage
Noradrenaline	Disulfiram, FLA-63**	Dopamine- $\beta$ -hydroxylase
5-Hydroxytryptamine	p-Chlorophenylalanine (pCPA) p-Chloroamphetamine (pCAM)	Rate limiting enzyme tryptophan hydroxylase
	Reserpine, Tetrabenazine**	Granular uptake and storage
	Fenfluramine, MDMA	Mechanism unknown
Histamine	$\beta$ -Hydrazino-histidine	L-Histidine decarboxylase

\* Peripheral depletory

\*\* Central depletor, Benserazide peripheral depletor in low dose, and both peripheral and central in high dose

MDMA – Methylenedioxymethamphetamine

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# Purinergetic and Adenosine Receptors

## PURINERGIC RECEPTORS

Burnstock and coworkers (1970,1972) reported that the transmitter substance released from the non-cholinergic and non-adrenergic nerves of the gut may be adenosine triphosphate (ATP) or some related purine nucleotide. These nerves have been designated as *purinergetic*. In most mammalian gut segments, both the nerve - mediated as well as ATP - induced responses are inhibitory, resulting in relaxation. In the mammalian bladder, these cause excitatory response producing contraction. In a number of tissues, such as guinea pig taenia coli, bladder, etc. regardless of whether the response is inhibitory or excitatory, the relative potencies of nucleotides and nucleosides usually give the same result (ATP > ADP > AMP > adenosine). In guinea pig ileum, there are both non-adrenergic inhibitory and non-cholinergic excitatory nerves, the stimulation of which may produce a biphasic response, usually a relaxation followed by contraction that can also be produced by ATP.

Adenosine uptake by purinergetic nerves is analogous to the uptake of choline in cholinergic transmission after degradation of released acetylcholine by acetylcholin-esterase, and also to the uptake of noradrenaline in adrenergic transmission. Dipyridamole and hexabendine by blocking this uptake potentiate the effects of both ATP and purinergetic nerve stimulation.

Pyridylisatogen, a specific ATP antagonist, fails to block the inhibitory response of the guinea pig taenia caeci to transmural stimulation, although it blocks the relaxation induced by ATP and ADP, but not by adenosine, noradrenaline, isoprenaline or AMP.

Purinergetic receptors, which are present in brain, peripheral tissues and blood cells, are divided into two large families, P1 (adenosine), and P2 (adenosine triphosphate or ATP) receptors. P1 receptors are further divided into subtypes,  $A_1$ - $A_2$ , and P2 receptors into P2X and P2Y. All the purinergetic receptors mediate their responses via G proteins, except P2X receptors, which are considered as ligand-gated ion channels. Only the P2Y receptors have been found to activate MAP kinase.

The characteristics of different subtypes of P1 and P2 receptors are presented in Table 13.1.

Table 13.1  
Characteristics of different subtypes of purine receptors

Criteria	P1 (Adenosine)				P2 (ATP)	
	A <sub>1</sub>	A <sub>2</sub>	A <sub>2b</sub>	A <sub>3</sub>	P2X	P2Y
Responses via G proteins	+	+	+	+	-	+
Ligand-gated ion channel	-	-	-	-	+	-
Response to adenosine and AMP	+++	+++	+++	+++	+	+
Response to ATP and ADP	+	+	+	+	+++	+++
Adenylyl cyclase	-	+				
Activate MAP kinase	-	-	-	-	-	+
Antagonized by xanthines	+	+	-	-	-	-
Antagonized by pyridylisatogen	-	-	-	-	+	+

### ADENOSINE RECEPTORS

The adenosine receptors, a class of purinergetic receptors, belong to G-protein coupled receptor (GPCR) superfamily. Activation of these receptors produces stimulation or inhibition of adenylyl cyclase activity leading to the corresponding changes in the cyclic AMP levels. Adenosine, the endogenous ligand, is a neuromodulator having a modulatory effect on central and peripheral neuronal activity increasing or decreasing the rate at which a nerve cell fires. It is used in the treatment of supraventricular tachycardia, and as a diagnostic tool in the investigation of cardiac abnormalities.

In humans there exist four subtypes of adenosine receptors:

1. *A<sub>1</sub>-Adenosine receptor* – present throughout the body it produces mainly inhibition of adenylyl cyclase activity although there may be coupling via G-proteins to ion channels and phospholipase C. It is thought to mediate the inhibition of transmitter release and the reduction in neuronal activity. It has an inhibitory action like slowing of metabolic activity in the brain. Presynaptically, it reduces synaptic vesicle release, and postsynaptically it stabilizes magnesium on the *N*-methyl *D*-aspartate (NMDA) receptors. Acting on this receptor the endogenous adenosine plays a role in regulating

myocardial oxygen consumption and coronary blood flow. Stimulation of this receptor brings about a decrease in the heart rate by decreasing the conduction of electrical impulses and suppressing pacemaker cell function. This makes adenosine an important tool in diagnosing and treating tachyarrhythmia. Blockade of this receptor, on the other hand, produces acceleration of heart beats. Caffeine and theophylline, the non-selective antagonists of adenosine receptors, are used to stimulate respiration in premature infants. 2'-MeCCPA is a very selective and potent  $A_1$ -receptor agonist, while PSB 36 and SLV 320 are potent and selective antagonists.

2.  $A_{2A}$ -Adenosine receptor – abundant in basal ganglia, vasculature and platelets. It modulates the stimulation of adenyl cyclase activity. The  $A_{2A}$ -receptor is supposed to influence dopamine-mediated responses in basal ganglia. The receptor on platelets produce anti-aggregatory effect. Like  $A_1$ -adenosine receptor the  $A_{2A}$ -receptor also plays a role in regulating myocardial oxygen consumption and coronary blood flow. Caffeine is a non-selective, while ZM281385 and SCH-58261 are potent and highly selective  $A_{2A}$ -antagonists. Specific agonists are CGS21680 and ATL-146e.
3.  $A_{2B}$ -Adenosine receptor – its function is not yet clear; may play some role in axon elongation. CV1808 is a non-selective agonist, while MRS1754 is a selective and PSB306 is a highly selective antagonist.
4.  $A_3$ -Adenosine receptor – the smallest receptor so far cloned modulates the inhibition of adenyl cyclase activity. It inhibits some specific signal pathways of adenosine which allows the inhibition of growth in human melanoma cells. Specific antagonists are MRS1523, MRS1191, PSB10 and PSB11, while selective agonist is MRS 3558, and highly selective agonist is 2-Cl-IB-MECA.

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# Opioid and Cannabinoid Receptors

## OPIOID RECEPTORS

Opiates and benzodiazepines are historical examples where the drugs were introduced long before the discovery of their receptors. Lee and Smith (1980) proposed that the opiate analgesic receptor is protein-lipid complex in nature consisting of two distinct classes of binding sites: a) protein sites for endogenous enkephalins, and b) lipid sites for alkaloids including morphine, while both sites for  $\beta$ -endorphin. The endogenous ligands for opioid receptors in mammals include three families of peptides – the enkephalins, endorphins and dynorphins. Methionine-enkephalin appears to act at a site distinct from that of leucine-enkephalin; the former inhibits the analgesic effect produced by large doses of morphine, while the latter potentiates the analgesic effect of the low doses of morphine. Martin and co-workers (1976, 1977) postulated the existence of three subspecies of opioid receptors with their respective agonists and antagonists.

The three types of G-protein-coupled opioid receptors are as follows:

1.  $\mu$ -Opioid receptors – mu-opioid receptors named after morphine, a pure  $\mu$ -receptor agonist. These receptors have high affinity for enkephalins and  $\beta$ -endorphin but low affinity for dynorphins. There are two subtypes of mu-receptor, (a)  $\mu_1$ -receptors, stimulation of which produce analgesia, and (b)  $\mu_2$ -receptors, stimulation of which lead to respiratory depression and constipation. Development of tolerance of different effects at different rates is due to the activation of these subtypes of mu-receptors. Alvimopan is a selective  $\mu_2$ -receptor antagonist used for treatment of postoperative ileus following bowel resection. Methylnaltrexone is another  $\mu_2$ -receptor antagonist used in the treatment of constipation associated with opioid analgesia. In animal studies the compound CX717 (AMPAKINE) has been shown to prevent or reverse opiate-induced respiratory depression without the loss of analgesia presumably by acting through  $\mu_2$ -receptors.  
Two peptides endorphin 1 and endorphin 2, with a difference in one amino acid between them, have been identified in mammalian brain. They exhibit the highest specificity and affinity for the  $\mu$  receptor. They may be considered as natural ligands for this receptor.
2.  $\kappa$ -Opioid receptors – kappa-opioid receptors named after ketocyclazocine, a pure k-receptor agonist. They bind with dynorphins, endorphins and enkephalins in a decreasing order of affinity.

3.  $\delta$ -Opioid receptors – delta-opioid receptors named not after any drug but after deferens of mouse vas deferens, where this receptor was found in high concentration. Delta-receptors have approximately equal affinity for endorphins and enkephalins, and less affinity for dynorphins.

The properties of different opioid receptors are summarized in Table 14.1

Table 14.1  
Properties of different opioid receptors

Criteria	$\mu$ -Opioid receptor	$k$ -Opioid receptor	$\delta$ -Opioid receptor
Receptor family	GPCR	GPCR	GPCR
Stimulation	Supraspinal analgesia Respiratory depression Euphoria, physical dependence	Spinal analgesia Miosis, sedation	Dysphoria, hallucinations Respiratory and vasomotor stimulation
Endomorphin-1	Highly selective	Less selective	Less selective
Morphine	Highly selective	Less selective	
Agonists	DAMGO*, Fentanyl* Endorphin, Methadone* Pentazocine (PA), Enkephalin Etorphine, Morphine* Buprenorphine (PA) Levorphanol	Pentazocine, Etorphine Spiradoline, Dynorphin A Cyclazocine, Levorphanol	Etorphine, Enkephalin, Cyclazocine, Levorphanol Endorphin, Deltorphin*
Antagonists	Naloxone, CTOP*, Naltrexone, Pentazocine, Nalorphine, Diprenorphine Alvimopan**	Naloxone, Naltrexone Buprenorphine, Diprenorphine, nor-Binaltorphimine (Nor-BNI)*	Naloxone, Naltrindole* Naltrexone

GPCR – G protein-coupled receptor

\* Selective

PA – Partial agonist

\*\* Selective for peripheral receptor in G.I. tract

### CANNABINOID RECEPTORS

The cannabinoid receptors (CB) belong to the G-protein coupled receptor family. They are activated by cannabinoids, generated naturally inside the body (endocannabinoids), or introduced into the body as cannabis or related synthetic compounds. Following the binding of the receptors by the ligands, multiple intracellular signal transduction pathways are activated.

There are two known subtypes of cannabinoid receptors:

1.  $CB_1$ - receptors present mainly in the brain; also in the liver, lungs and kidneys. Activation of presynaptic  $CB_1$ - receptors inhibits sympathetic innervation of blood vessels, while activation of those in the liver increases de novo lipogenesis. Endocannabinoids released from the depolarized neuron bind to  $CB_1$ - receptors in the pre-synaptic neuron leading to a reduction in GABA release.

2. CB<sub>2</sub> - receptors are mainly present on T cells of the immune system, on macrophages and B cells, and in haemopoietic cells. They are also present in peripheral nerve terminals; in the brain they are mainly present in microglial cells.

In addition, there are possibilities of existing non-CB<sub>1</sub> and non-CB<sub>2</sub> receptors in endothelial cells and in the CNS. Cannabinoids bind reversibly and stereoselectively to the CB receptors. Cannabinoids that bind more selectively to certain receptors may be exploited for medicinal usage. Tetrahydrocannabinol (THC) as well as two major endogenous compounds – anandamide and 2-arachidonylglycerol (2-AG) produce most of their effects by binding to both the CB<sub>1</sub> and CB<sub>2</sub> receptors. Administration of Δ<sup>9</sup>-THC, or anandamide produces CB<sub>1</sub>-mediated inhibition of gastrointestinal activity; specific CB<sub>1</sub> antagonist SR141716A (Rimonabant) blocks this effect. The inhibition of intestinal motility, however, may also have a CB<sub>2</sub> mediated component. Activation of peripheral CB<sub>1</sub> receptors is responsible for hemorrhagic and endotoxin-induced hypotension that may be mediated by anandamide produced by macrophages, and by (2-AG) produced by platelets. Anandamide by interacting with CB<sub>1</sub> receptors located on peripheral sensory nerve endings attenuates the pain behaviour in formalin treated animals.

Cannabinoid (CB<sub>1</sub>) antagonists have been shown to have protective effect against doxorubicin-induced cardiotoxicity. Doxorubicin (DOX) is one of the most potent antitumour agent whose clinical use is limited by its severe cardiotoxicity. Rimonabant, a CB<sub>1</sub> receptor antagonist, blocks the CB<sub>1</sub> receptor selectively. It has been shown to decrease food intake, and to regulate body weight gain.

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# Angiotensin, Prostaglandin and Nitric Oxide

## ANGIOTENSIN

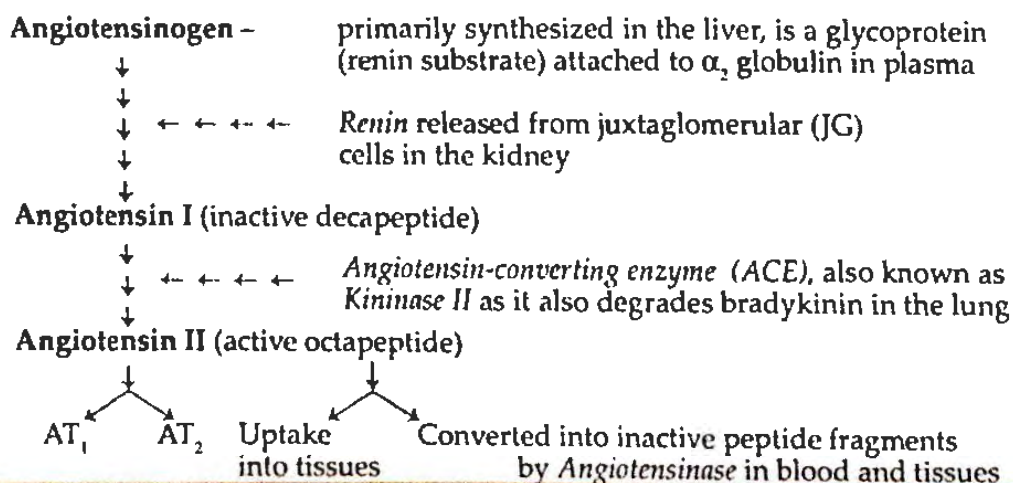
Two subtypes of angiotensin II receptors have been identified and designated as  $AT_1$  and  $AT_2$  (Bumpus *et al.* 1991). These receptors are distributed in the brain, heart, vasculature, adrenal gland and kidney. Both the subtypes belong to the G protein-coupled receptor (GPCR) family (Murphey *et al.* 1991). Most of the biological effects of the active form of angiotensin (Angiotensin II) such as a rise in the blood pressure, an increase in the aldosterone secretion, and cell proliferation are mediated through the  $AT_1$  receptor. The functional role of the  $AT_2$  receptor is ill understood although the recent studies indicate its possible role as antiproliferative, apoptotic (programmed cell death) and vasodilatory functions.

### Angiotensin II Receptor Blockers

$AT_1$  receptor exhibits a high affinity (more than 10,000-fold) for biphenylmethyl derivatives, such as losartan, candesartan, valsartan, irbesartan and telmisartan; candesartan being the most effective, while losartan the least (Mimran *et al.* 1999). Although the blocking is of competitive nature, the inhibition of biological responses to angiotensin II is often insurmountable. The  $AT_1$  receptor has a low affinity for the compound PD 123177. The  $AT_2$  receptor, on the other hand, has a high affinity for PD 123177, and a low affinity for losartan and related compounds.

### Angiotensin II and its Formation and Fate

It is the most potent pressor substance, being about 40 times more potent than noradrenaline on a molar basis. The formation and fate of angiotensin II are as follows.



Renin-release is blocked by  $\beta$ -adrenergic blockers at JG cells, and by clonidine decreasing secretomotor impulses from the central nervous system to JG cells.

ACE, which catalyses the synthesis of angiotensin II and destruction of bradykinin in lung, is blocked by teprotide, a nonapeptide bradykinin potentiating factor (BPF), and by captopril, an orally effective nonpeptide (Rubin *et al.* 1978).

Saralasin (1-sarcosine, 8-isolucine angiotensin II), an angiotensin II analogue, is a specific blocker of angiotensin II receptor, and also a partial agonist.

### Assay of Angiotensin

Angiotensin is assayed by its pressor effect on the rat blood pressure rendered sensitive by vagotomy and ganglion blockade. It can also be assayed on the rat isolated ascending colon that is rendered specific to angiotensin by the use of a mixture of antagonists, viz. oxprenolol  $10^{-5}$ , phentolamine  $10^{-6}$ , atropine  $10^{-6}$ , methysergide  $5 \times 10^{-7}$ , and polyphloretin phosphate  $2 \times 10^{-5}$  g/ml. The rat ascending colon, which is sensitive to angiotensin, is relatively insensitive to substances likely to be found in the blood, viz., histamine, oxytocin, antidiuretic hormone and bradykinin. The specific inhibitor, 8-L-ala-angiotensin-II,  $2.5 \times 10^{-7}$  g/ml may be used as a final proof for angiotensin (Gagnon and Sirois, 1972).

## PROSTAGLANDIN

A group of compounds known as prostaglandins were originally isolated from the seminal fluid, now shown to be present in many tissues in the body. They are C20 unsaturated fatty acids containing two or more double bonds and hydroxyl groups and a five-member ring related to arachidonic acid. The parent fatty acid is known as prostanoic acid. Prostaglandins of the E and F series, which are most abundant and most intensively studied, are referred to as primary prostaglandins.

### Biosynthesis of Prostaglandins (Table 15.1).

In man, arachidonic acid is either derived from dietary linoleic acid, or is ingested as a constituent of meat. It is then esterified as a component of cell membrane, or found in ester linkage in other complex lipids. Arachidonic acid is released from membrane phospholipids by the action of the enzyme phospholipase  $A_2$ . This in turn is converted to endoperoxides by widely distributed fatty acid cyclo-oxygenase, and to the corresponding hydroperoxides by lipo-oxygenase present in lung, platelets and white cells. Isomerization both enzymatically and nonenzymatically of endoperoxides leads to the synthesis of primary and other prostaglandins.

#### Inhibitors of Prostaglandin Biosynthesis

1. *Inhibition of the release of arachidonic acid from the membrane phospholipids by inhibiting the enzyme phospholipase  $A_2$ : p-bromophenacyl bromide (PBPAB) (Mitchell *et al.* 1977).*



2. *Competitive inhibition of prostaglandin production by false substrate*: acetylenic analogues of arachidonic acid, e.g. 5, 8, 11, 14-eicosatetraenoic acid (normal substrate 5, 8, 11, 14-eicosatetraenoic acid).
3. *Inhibition of cyclo-oxygenase enzyme thereby preventing the production of prostaglandin endoperoxides by nonsteroidal anti-inflammatory drugs (NSAIDs)*: aspirin, indomethacin, sodium salicylate, etc. Inhibition of this enzyme may also enhance production of slow reacting substance of anaphylaxis (SRS-A) responsible for aspirin-sensitive bronchospasm in asthmatic patients (Foegh, 1979). In general, tissues do not store prostaglandin; hence the release indicates fresh synthesis. Both synthesis and release are inhibited by NSAIDs.
4. *Inhibition of the formation of  $PGI_2$  in vitro*: lipid peroxides, e.g. 15-hydroperoxyarachidonic acid and their methyl esters.
5. *Inhibition of thromboxane synthetase*: imidazole and some of its analogues, e.g. phenylphosphonate compound N-0164.

### Metabolism

The pharmacological activity of prostaglandins E and F (PGEs and PGFs) is almost entirely reduced in their passage through the lungs or by incubation with lung extracts. Inactivation of PGs can be inhibited by low concentrations of di-4-phloretin phosphate (DPP) and polyphloretin phosphate (PPP) (Crutchley and Piper, 1975).

### Assay of Prostaglandin

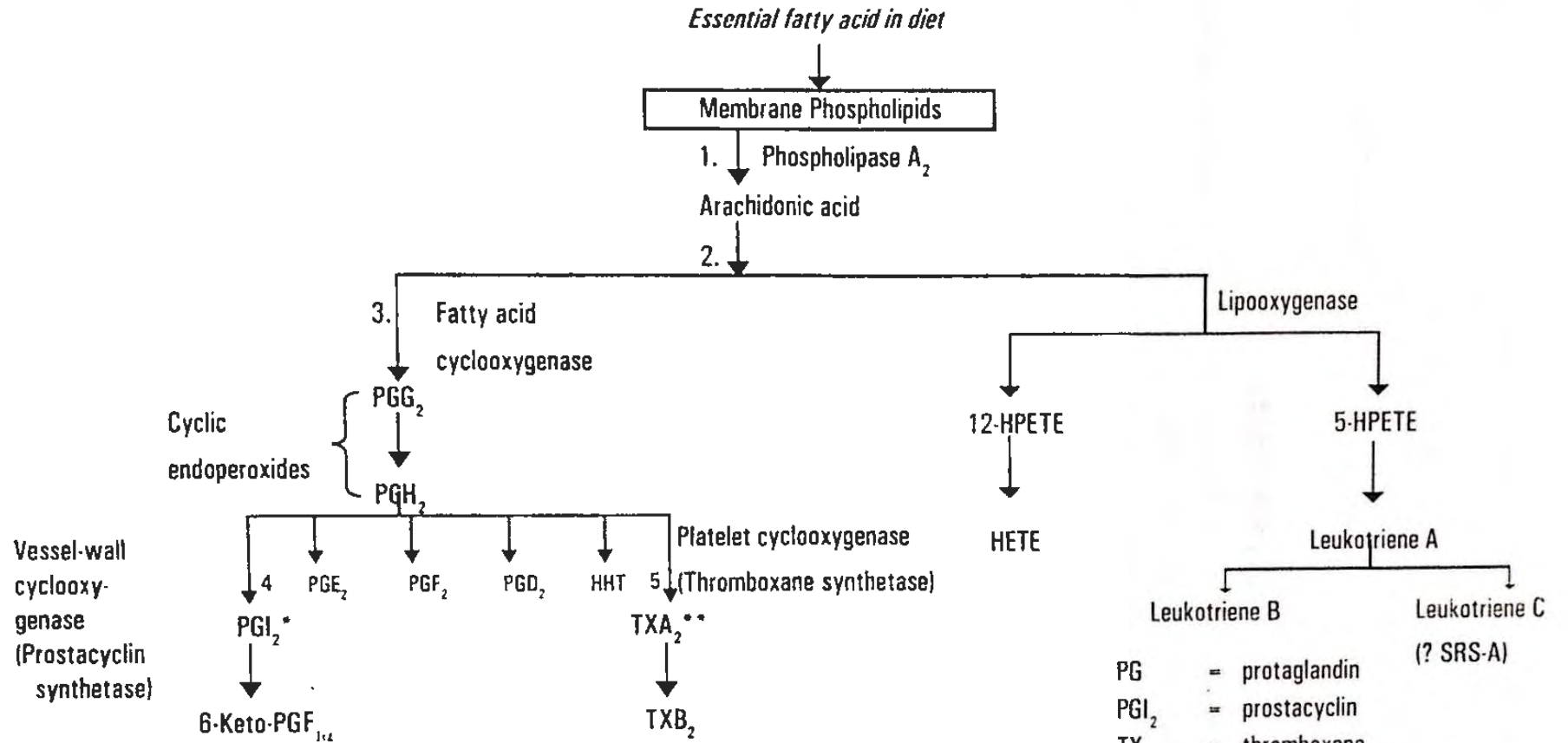
Rat stomach fundus strip may be used for the assay of  $PGE_2$  in Kreb's solution gassed with carbogen, and containing a mixture of selective antagonists, hyoscine  $0.33 \mu\text{M}$ , mepyramine  $0.35 \mu\text{M}$ , methysergide  $0.57 \mu\text{M}$ , phenoxybenzamine  $0.33 \mu\text{M}$  and propranolol  $11.57 \mu\text{M}$ .

Rat colon strip may be used for  $PGF_{2\alpha}$ .

Hamster stomach strip is relatively insensitive to 5-hydroxytryptamine and histamine; hence, suitable for the assay of prostaglandins in body fluids and tissue extracts. It is relatively more sensitive to prostaglandin  $E_2$  than  $F_{1\alpha}$  (Ubatuba, 1973).

Prostaglandins may first be separated by differential extraction or by chromatography. PGFs may be assayed selectively by first inactivating the PGEs by treatment with alkali. The majority of the primary prostaglandins can be distinguished on preliminary screening by employing rat stomach strip, rabbit coeliac artery and rabbit transverse stomach strip (Moncada *et al.* 1977). Assays of  $PGE_2$  and  $PGF_{2\alpha}$  in mixtures can be carried out by using two tissues, the rat fundus and the rat colon.  $PGE_2$  contracts rat fundus but not colon, whereas  $PGF_{2\alpha}$  contracts rat colon, but is much weaker than  $PGE_2$  on rat fundus.

**Table 15.1**  
**Biosynthesis of prostaglandins†**



\* Anti-aggregatory vasodilator PGI<sub>2</sub> production decreased by high concentration of aspirin, thus enhancing platelet aggregation.

\*\* Pro-aggregatory vasoconstrictor TXA<sub>2</sub> production decreased by low concentration of aspirin resulting in anti-aggregatory activity

PG = prostaglandin  
 PGI<sub>2</sub> = prostacyclin  
 TX = thromboxane  
 HHT = 17-carbon hydroxy acid  
 HPETE = hydroperoxyeicosatetraenoic acid  
 HETE = hydroxyeicosatetraenoic acid  
 SRS = slow reacting substance  
 For explanation of numerals see text.

† Moncada, S., Flower H. J. and Vane, J.R. (1980) In *Goodman and Gilman's The Pharmacological Basis of Therapeutics*, eds. Gilman, A.G., Goodman L.S. and Gilman, A., 6<sup>th</sup> edn., p.668, Macmillan Publishing co., Inc., New York.

## Antagonists

SC-19220, a dibenzoxazepine hydrazide derivative, is a relatively specific competitive antagonist of PGs on smooth muscle preparations. PPP and DPP also have similar antagonistic action on smooth muscles. Flufenamic acid and meclofenamic acid block the isolated bronchial smooth muscle contractions induced by  $\text{PGF}_{2\alpha}$ . N-0164 is a potent partially selective prostaglandin antagonist on smooth muscle preparations.

### NITRIC OXIDE

Nitric oxide (NO), chosen as the "molecule of the year" by the Science Magazine in 1992, has been found to control a wide range of bodily functions including blood pressure, activities of the brain like sending messages and storing memories, and blood flow to vital organs. It also acts as a neurotransmitter diffusing all over thereby acting on several nearby neurons, even those not connected by synapses. The immune system uses it to fight all types of infections as well as tumours.

In the year 1980, Furchgott and Zawadzki observed that the relaxation of blood vessels by acetylcholine is due to the release of a compound by endothelial cells acting on smooth muscle cells of blood vessels. This compound they named "endothelial derived relaxing factor or EDRF". In 1986, Furchgott and Ignarro and their associates independently proposed that EDRF was really nitric oxide. Palmer *et al.* (1987) demonstrated that endothelial cells produced nitric oxide in sufficient amounts to explain the relaxation of blood vessels observed by Furchgott and co-worker.

The tiny molecule nitric oxide (NO), a reactive gas, functions both as signaling molecule in endothelial cells and nerve cells, and as a killer molecule by activating immune cells. Nitric oxide is not only a potent vasodilator but also inhibits platelet adherence and aggregation, reduces adherence of leukocytes to the endothelium, and suppresses proliferation of vascular smooth muscle cells.

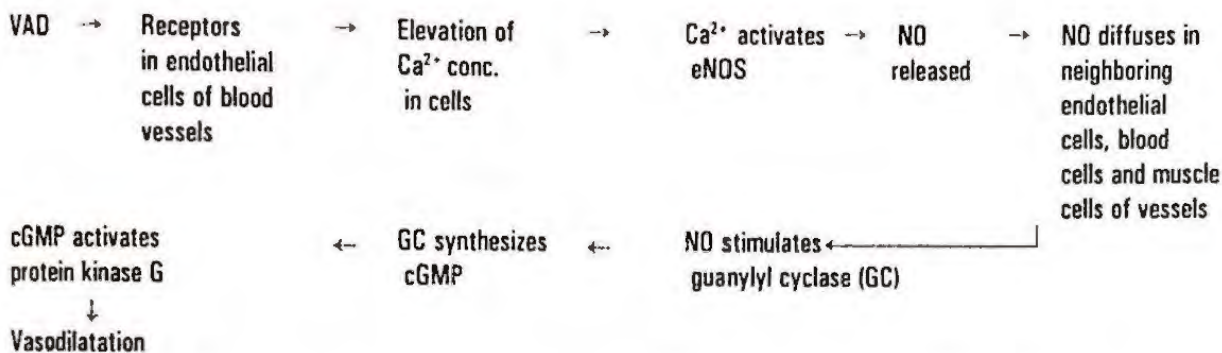
#### Biosynthesis of Nitric Oxide (NO)

Nitric oxide is synthesized within the cells by oxidation of L-arginine to NO and L-citrulline by a family of enzymes known as nitric oxide synthases (NOS). Three isoforms of NOS have been identified and named after the cell types where they are found. These are: (a) neuronal NOS (nNOS or NOS I) found in neurons, (b) inducible NOS (iNOS or NOS II) found in macrophages, and (c) endothelial NOS (eNOS or NOS III) found in endothelial cells lining blood vessels.

All these subtypes of NOS utilize L-arginine, oxygen, and NADPH as substrates, and produce nitric oxide, L-citrulline, and  $\text{NADP}^+$ , while FMN, FAD, tetrahydrobiopterin, heme and  $\text{Ca}^{2+}$ /calmodulin act as cofactors. The biological actions of NO are terminated by its spontaneous oxidation to  $\text{NO}_2$  and  $\text{NO}_3$ , its biological half-life being 3 to 5 sec. This allows NO to function locally as an autocoid.

## Role of NO in producing vasodilatation

The various steps by which the vasoactive drug (VAD) produces vasodilatation are as follows:



N.B. Nitroglycerine is converted to NO inside vascular tissue, which causes vasodilatation and inhibition of blood clotting.

### NOS inhibitors:

Aminoguanidine, dexamethasone, and a number of arginine derivatives, like N<sup>G</sup>-monomethyl arginine (NMMA) are potent inhibitors of NOS.

The cytotoxic effect of endotoxin on vascular endothelial cells is inhibited by NMMA, and by dexamethasone & hydrocortisone, the two inhibitors of the induction of nitric oxide synthases (NOS), suggesting the cytotoxic effect of endotoxin is mediated by the nitric oxide synthesized by inducible NOS. This may also be a mechanism for the local endothelial damage during endotoxin shock and other immunologically based conditions.

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# Studies on Isolated Muscle Preparations

The advantages of isolated tissues over intact animals are that several preparations can be obtained from a single animal; relatively small amount of the test material is required; and the drug effect is tested directly without the factors of absorption, metabolism, excretion or interference due to nerve reflexes. Because of these, the results obtained on isolated experiments are not always reproducible when tested on the whole animal.

Guinea pigs, rabbits and rats are usually the common sources of isolated tissues. Mice are also sometimes used, while cats and dogs are too big to be sacrificed just for a piece of tissue. In the latter cases, the preparations are generally obtained from anaesthetized animals used for other tests.

The isolated strips of intestine are the most commonly employed smooth muscle preparations because (i) the materials are abundant, (ii) they are more resistant to handling, (iii) they are relatively easy to set up, (iv) different sections have a variable degree of spontaneous activity, and (v) they permit the study of different types of pharmacological actions. Ileum being relatively more resistant to trauma is easier to set up and produces larger contractions than jejunum or duodenum. The functional components of the isolated intestines are terminal sympathetic and parasympathetic synapses as well as parasympathetic ganglionic synapse. Guinea pig and rabbit intestines are routinely used in preference to other species.

## Muscle Tone

Some isolated muscle preparation exhibits a sustained submaximal contraction or inherent tone, which may be of neurogenic or myogenic origin or of both. It represents the background activity of the muscle. When the degree of tone is high, as in the case of rabbit duodenum and jejunum, rat stomach strip, guinea pig taenia caeci and ascending colon, the smooth muscle may respond by relaxation following a particular drug. Intramural prostaglandin production contributes to this inherent tone of the muscle (Ferreira *et al.* 1976). Oxygen or glucose lack may abolish this tone by interfering either with the intramural generation of, or with the reaction of the tissue with prostaglandin.

## Biophase

The environment in which a drug is in position to interact with the receptors without intervening diffusion barriers is known as biophase. In most of the isolated

tissue preparations, the concentration of drug in biophase is in equilibrium with the surrounding physiologic salt solution. In case of many drugs, when there are sites of loss in intimate association with the receptors (e.g. due to neuronal uptake mechanism), there may be marked difference in the drug concentration between the biophase and the salt solution unless the site of loss is blocked (Waud, 1968).

### Contact Period of the Drug with the Tissue

The contact period of a water soluble drug with the tissue is regulated depending on the time the response reaches a steady level. If the response is slow, or when a poor water soluble drug is added to the bath in the form of a suspension, the contact period with the tissue should be kept constant, since the effect may increase with longer contact period. The usual effect of drugs on isolated intestines is produced by penetration through the serosa and not through the mucosa. It should be remembered that agonists can be rapidly rinsed out from a tissue, but antagonists may take longer time to leave a tissue despite repeated washings.

### Rate of Action of Drugs

The rate of action of drugs will depend on: (a) access to the receptor through diffusion barriers, (b) conversion of the drug from an inactive to an active form, (c) rate of combination with the receptor, and (d) rate of production of response, viz. fast contraction by guinea pig ileum or slow contracture by frog rectus abdominis muscle.

## EXPERIMENTAL PROCEDURES

Before sacrificing the animal, all equipments should be checked for their perfect working condition. The organ bath should be scrupulously cleaned, filled with the physiologic salt solution bubbled with gas, and maintained at desired temperature. The writing lever should be adjusted for the desired degree of magnification and tension.

Small animals are usually killed by a head-blow (stunning), and organs to be studied removed rapidly and immersed in a salt solution in a shallow Petri dish. After putting up the tissue and giving rest for about 30 to 90 minutes, if the strip is found to relax, the lever is readjusted to horizontal position. Occasionally due to increased tonus, the strip may fail to relax until the tension on the lever is increased by readjustment of weights.

The organ bath is emptied at regular intervals, and the fluid is replaced by fresh solution during the experimental period to avoid alteration of pH due to prolonged aeration, which in turn changes the tone of the muscle.

The drum is started at a slow speed. After measuring the volume of the drug solution accurately (0.1 to 0.5 ml) with the help of a 1 ml graduated pipette, or a tuberculin syringe fitted with a needle, the drug is added to the bath fluid with uniform speed. Simultaneously with the addition of the drug a stopwatch is

started. The drug is allowed to act till the response reaches a steady level, or up to a fixed time (say, 30 to 60 sec) depending on the nature of the experiment, after which the bath fluid containing the drug is washed out and fresh solution is allowed to fill the bath. The washing is repeated two or three times in order to allow the lever to come back to the original base line. At the time of washing, the drum is stopped so that any contraction due to the momentary exposure of the tissue to air is recorded as a vertical kick readily discernible from true contractions. This can, however, be avoided by emptying the organ bath by the overflow method, that is, by replacing the fluid with fresh solution from the bottom without actually emptying the bath. After some definite interval, depending on the tissue as well as the drug, another dose of the drug is added and the stop watch is restarted, thus completing a cycle which is strictly followed and repeated throughout the whole experimental period. A spontaneous activity especially of the uterus is sometimes overcome by lowering the bath temperature or by reducing the interval between successive doses. Sensitivity of the preparations may sometimes be improved by raising the temperature or by increasing the dose intervals.

Every time a drug is added into the bath, a mark is put on the drum near the base line and labeled appropriately indicating the drug used and its dose or concentration. Sometime, it is useful to include a time tracing below the base line. At the end of the experiment, the date, tissue employed, bath fluid, temperature, gas and the nature of the experiment, etc. are recorded on the smoked surface before fixing the tracings permanently.

The test should start with a very low concentration of the substance producing slight or no response, and gradually increasing the concentration 5-10 times more than the preceding one until the activity range is found, or until total ineffectiveness is proved by giving sufficiently large concentration. A sudden exposure of the tissue to a high concentration of drug may affect it for a prolonged period making it unsuitable for further testing. It is a good practice to try two or three standard drugs (e.g. acetylcholine, histamine, 5-hydroxytryptamine, etc.) before and after each addition of the unknown drug so as not to lose sight of the latter's inhibitory property, if any. In the case of an inhibitory drug, a very high concentration will have a nonspecific antagonism against a number of agonists, thus its true specific nature will not be detected.

### ISOTONIC AND ISOMETRIC CONTRACTIONS OF MUSCLE

Isotonic contraction of a muscle is the shortening of the muscle during contraction that can be recorded isotonicly under a steady load. Isometric contraction, on the other hand, is the development of tension (force) during contraction that is recorded isometrically without allowing the muscle to shorten. Auxotonic method of recording is that in which the restoring force increases in proportion to the extent of shortening. This may be done with a spring lever or



with an auxotonic pendulum lever, the restoring force being exerted by the spring or the pendulum.

The isotonic and the isometric responses coincide in some muscles, but differ greatly in other muscles. For instance, in frog rectus abdominis and in dorsal leech muscle, the percentage of shortening is much greater than development of tension by a given concentration of an agonist; while in guinea pig ileum longitudinal muscle, there is no difference between the percentage of shortening and development of tension. Michelson and Shelkovnikov (1976) compared quantitatively the ratio "EC50 isometric / EC50 isotonic" of agonists on different muscles and obtained the values as follows: frog rectus abdominis 5.0, leech dorsal muscle 4.3 and guinea pig ileum 1.0.

### STUDY OF THE MECHANISM OF DRUG DISPOSITION IN SMOOTH MUSCLE

The rate of relaxation of vascular smooth muscle *in vitro* is a function of the residual concentration of the active drug in the environment of tissue receptors. After a steady state, contraction is produced by an agonist in an aqueous medium; the bathing fluid is then replaced by mineral oil (liquid petroleum) to eliminate loss by diffusion out of the tissue. The relaxation curve is then recorded. This provides a direct measure of the termination of action by intrinsic mechanisms (Kalsner and Nickerson, 1968).

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# Some Standard Isolated Muscle Preparations

## GUINEA PIG ILEUM

Healthy adult guinea pig is stunned by head-blow, neck vessels cut, and the animal is bled out. Abdomen is opened through a midline incision, the ileocaecal junction exposed, the terminal ileum is cut after discarding 10 cm nearest to the ileocaecal junction because of the presence of excitatory  $\alpha$ -adrenoceptors near the ileocaecal junction (Munro, 1953). The mesenteric attachment is cut as close to the gut as possible without injury for a distance of about 20 to 25 cm. The intestine is then cut across, and the lumen of the isolated piece thoroughly cleaned by running warm salt solution repeatedly through the proximal opening, with the help of a 50 ml volumetric (bulb) pipette. Undue stretching, ballooning or handling of the gut should be avoided. The clean strip of the intestine is then placed in fresh warm salt solution for a short period for acclimatization before being put up for the experiment. If strips are to be kept for future use, they are better placed directly in ice-cold aerated salt solution, and kept in refrigerator overnight. About couple of hours before use, they are transferred to salt solution at room temperature and actively aerated. A small segment usually 4 to 6 cm in length (depending on the size of the organ bath) is cut; at each end a thread is passed through the lumen and the wall near the mesenteric attachment with the help of a fine sewing needle, and tied securely without occluding the lumen. To facilitate cutting out a segment of desired length, a scale (cm) may be marked on one side of the cork mat, on which these manipulations can be made with the tissue soaked in salt solution. One end of the segment is tied securely to the tissue holder or some other suitable contrivance, and transferred to the organ bath (already filled with salt solution and bubbled with gas) holding both the tissue-holder and the long thread attached to the tissue in the same hand to avoid accidental stretching. The tissue-holder is fixed in position with clamps and the long thread from the tissue is fixed to a frontal writing lever adjusted suitably for tension and magnification. For maximum sensitivity, the lever should be nearly balanced, and friction at the writing surface reduced to a minimum by using a smooth writing point. A small vibrator attached to the lever support to the base of the drum, or to the water bath may be used to reduce the effect of friction on the drum.

The preparation responds to most of the common spasmogens, and is particularly suitable for the assay of histamine.

**GUINEA PIG MYENTERIC PLEXUS – LONGITUDINAL MUSCLE\***

A piece of terminal ileum 10 cm long is slid on to a glass rod (5 to 6 mm dia). After setting the mesenteric attachment in a straight line, the longitudinal muscle is separated from the circular muscle by stroking firmly and tangentially into the attachment along the whole length of the segment and the whole circumference of the ileum with a wisp of cotton wool soaked in Krebs solution. The strip consists of all the longitudinal muscle with myenteric plexus adhered firmly to it. The mesenteric attachment seen as a whitish material is removed by taking one end with a fine forceps and pulling it off the muscle. A strip of suitable size is put up in an organ bath containing Krebs solution at 36 °C and bubbled with 5% CO<sub>2</sub> in O<sub>2</sub>. An auxotonic lever with 0.3 g load is used for recording of the muscle responses.

**HYPOGASTRIC NERVE – VAS DEFERENS PREPARATION OF THE GUINEA PIG\*\***

Guinea pig is killed by stunning and bled; the abdomen opened along the midline, and the gut displaced on one side. The testis is pushed into the abdominal cavity by pressure on the scrotum. Holding the testis, the vas deferens of one side is freed from the connective tissues and cut from the epididymis. Grasping the cut end of the vas deferens with small forceps, it is separated from the adjacent tissue and left as it is. The hypogastric nerves (right and left) are now identified in the middle of the mesentery of the colon. One nerve is tied and cut 5 cm from the vas deferens; this is cleaned to within 0.5 cm of the organ. A piece of peritoneum containing the remainder of the nerve up to the vas deferens is isolated. The vas deferens is then cut from the urethra and removed together with its nerve along with the small piece of peritoneum and put up in an organ bath containing Krebs solution. During the dissection, the organ and the nerve are moistened with Krebs solution. The contralateral preparation can also be dissected out, if required.

**GUINEA PIG TRACHEAL CHAIN**

Trachea is removed from a freshly killed guinea pig (500 to 600g) and sectioned with a pair of scissors into 10 to 12 approximately equal size rings. Rings are kept moist in Ringer's solution while they are being tied in series by short loops of silk thread. The rings are oriented in such a way that the dorsal smooth muscle band is in a line. In an alternative but simpler method, the trachea is cut spirally, or opened with a longitudinal cut along the mid-dorsal surface, and a series of transverse cuts made successively from alternate sides so that they overlap one another but do not transect the preparation completely.

\* Kosterlitz *et al.*, 1970

\*\* Hukovic, 1961

### GUINEA PIG TAENIA CAECUM

Guinea pig is killed by stunning and bled, and the abdomen is opened along the midline. The caecum is exposed, and a single strip of taenia is dissected out and kept in McEwen's solution. A length of 2-3 cm of the preparation is put up in 10 ml organ bath containing McEwen's solution at a temperature of 37°C and bubbled with 5% CO<sub>2</sub> in O<sub>2</sub>. Responses are recorded on a smoked drum with an isotonic frontal writing lever (tension 1 g., magnification 6-fold).

The preparation is suitable for the study of cholinergic agents and their antagonists both on muscarinic and on nicotinic receptors. Preparation with a low tone is most sensitive to acetylcholine, while that with high tone is least sensitive.

### RABBIT ILEUM OR JEJUNUM

These are put up in a similar fashion as the guinea pig ileum, the organ bath usually being larger in size. These are particularly suitable for producing barium or acetylcholine induced spasms for the study of their antagonism. Stored for four days at 4°C, rabbit ileum becomes increasingly sensitive to the inhibitory activity of isoprenaline.

### RABBIT DUODENUM

A strip of duodenum 4 to 5 cm long is removed from a freshly killed rabbit and put up in a similar way as the guinea pig ileum. This preparation is particularly suitable for adrenaline and noradrenaline that produce relaxation. The important factor for the success is the proper adjustment of the load or tension on the muscle. It is desirable that the strip should acquire a certain degree of tone, so that when it is relaxed by adrenaline the lever falls by 3 to 4 cm from the base line. If the weight on the lever is too much, no tone develops although there may be rhythmic contractions. The only effect of adrenaline is then to decrease the size of the rhythmic contractions. The weight on the lever, on the other hand, must be enough to cause it to fall when the tone is reduced by adrenaline. Hence, it is essential to find out the optimal weight on the lever by trial and error even if it involves spending some time. The segment is then left in the bath for some time in order to develop the requisite tone; repeated addition of adrenaline with washing facilitates this process further. In the course of half to one hour, the strip should acquire sufficient tone so as to enable the actual assay to be performed.

While the peristaltic movements depend on the ganglion cells, the rhythmicity or the pendular movement is inherent only in the muscle.

### RABBIT AORTIC STRIPS\*

A rabbit (2.5 to 3.5 kg) is stunned and exsanguinated (decapitated). The

\* Furchgott and Bhadrakom, 1953

descending thoracic aorta is removed and placed in Krebs-bicarbonate solution. Excess fat and connective tissues are trimmed off, and the whole length of the aorta is then cut in a close spiral as follows. The aorta is held horizontally at one end with the left hand and is gradually rotated and moved forward while a continuous spiral (helical) cut is made with a small sharp-pointed scissors. The resulting strip is usually about 2 mm wide and 20 cm long with smooth circular fibres oriented at about 15 degrees relative to its long axis. From this strip shorter strips (2 to 4 cm) are cut for the use in the experiments. The tissue is kept moistened in Krebs solution during the whole procedure. The strip is mounted in an organ bath and the preparation is allowed to stabilize for 90 minutes before the actual experiment is started. During this period the bath fluid is changed every 15 minutes.

The preparation has a very slight spontaneous tone and shows no rhythmic contraction. It responds to  $\alpha$ -adrenoceptor agonists like noradrenaline and adrenaline by contractions. Histamine and acetylcholine also produce contractions but in much higher concentrations.

#### RABBIT PERFUSED EAR ARTERY\*

Lop-eared rabbit (2.5 to 4 kg) is anaesthetized with 25% urethane 7 ml/kg i.p. The hair over the base of the ear and along the course of the central artery is clipped and the skin is incised adjacent to the central artery and vein, which are dissected carefully avoiding damage to these structures. The central artery is identified at the base of the ear; ligatures are placed around it preparatory to cannulation. The ventral auricular nerve that runs parallel to the central vein is dissected free and ligated. Heparin (1000 units) is injected i.v; the artery is then cannulated after a couple of minutes with fine polythene tubing about 1 cm of which is inserted into the artery, and the ligature tied over it. The central artery together with the adjacent structure including the central vein and the ventral auricular nerve are dissected free from the cartilaginous bed of the ear for a length of 4 to 7 cm and width of about 4 mm. The arterial segment is perfused with Krebs-bicarbonate solution prewarmed at 37°C bubbled with 5% CO<sub>2</sub> in O<sub>2</sub>. The perfused tissue is suspended in Krebs solution at 37°C; the effluent solution is washed off from the bath by an overflow tube. The perfusion pressure is measured by means of an ordinary or a Condon mercury manometer recording on a kymograph.

Stimulation transmurally, periarterially, or through the ventral auricular nerve produces vasoconstriction.

Drugs are injected through a rubber connection close to the cannula in a volume of 0.05 to 0.1 ml freshly prepared in Krebs solution. The preparation responds by constriction to noradrenaline in nanogram doses, and to histamine and 5-hydroxytryptamine in microgram doses. Acetylcholine produces dilatation only when the tone of the artery is raised.

\* de La Lande and Rand, 1965.

## Some Standard Isolated Muscle Preparations

The preparation may be utilized for the bioassay of catecholamines, and for the comparison of adrenergic neuron blocking drugs.

### RAT COLON

The first 4 cm of the descending colon (distinguished by diagonal strips of muscle on its surface) is used. This tissue provides a sensitive test organ for noradrenaline that generally has slightly more effect than adrenaline. Storing the tissue for 24 hour at 4°C can further increase the sensitivity to noradrenaline; this process also minimizes the spontaneous movements.

The rat colon is also highly sensitive to substance P and to some extent to angiotensin and to  $\text{PGF}_{2\alpha}$ .

### RAT DUODENUM

Proximal 3 cm of duodenum from freshly killed rat (150 to 250 g) is dipped in salt solution at 4 to 6°C for two to three hours before use. It is suitable for the identification of bradykinin that relaxes the preparation in very minute concentration (more than 1000 times sensitive compared to other relaxants), while substance P contracts it. The dose contact of drugs in this preparation is usually 30 seconds at intervals of three to five minutes.

### RAT STOMACH\*

The stomach from a freshly killed rat is dissected out and placed in the salt solution. The translucent fundus (rumen) is cut along the pylorus (thick and red) leaving a thin band of the pyloric tissue attached to the fundus, and its contents washed clean. The fundus is then cut open along the lesser curvature and spread on a cork mat soaked in salt solution. Alternative transverse cuts are made to preserve the longitudinal muscle. The strip is then pulled out by cotton thread tied on each end, and protrusions and fringes of pyloric tissue trimmed away to give a long clean thin strip for suspension in the bath.

It is suitable for the assay of 5-hydroxytryptamine being very sensitive to it. It is 10 times less sensitive to acetylcholine, the effect of which can be blocked by hyoscine. It is over 1000 times less sensitive to histamine. It is also used for the assay of  $\text{PGE}_2$ .

### RAT UTERUS

Although the guinea pig uterus was used in classical experiments on anaphylaxis by Dale, and for the assay of pituitrin for some years, this has since been replaced by the rat uterus, which produces relatively less spontaneous contraction.

The rat uterus is chiefly used for the assay of oxytocin, 5-hydroxytryptamine and adrenaline. For the estimation of oxytocic activity, the uterus from the virgin

\* Vane, 1957

rat (140 to 200 g) is dissected out and the two horns divided. One horn is suspended in the bath and the other horn kept in salt solution at room temperature for later use, if needed. By using de Jalon's solution the rhythmic contractions are abolished.

To have relatively quiescent but sensitive uterus for routine assays, the virgin rat (120 to 200 g) is injected with stilboestrol 24 hour before sacrificing. For 5-hydroxytryptamine assay, a relatively large dose of stilboestrol (0.25 mg/100 g) is injected intraperitoneally for three days before sacrificing. As little as 2 ng of 5-hydroxytryptamine per ml of bath fluid can be detected in this preparation. The acetylcholine response is blocked by atropine. Histamine by acting on  $H_2$ -receptor releases noradrenaline, which in turn relaxes uterine muscle. It is very sensitive to stimulation by posterior pituitary extract, bradykinin, substance P and adenosine compounds, and to inhibition by adrenaline and noradrenaline.

One of the most specific and sensitive tests for adrenaline is the use of the rat uterus, which contains only  $\beta$ -receptors. Contractions are produced at regular intervals with acetylcholine or carbachol, and adrenaline estimated by its power of reducing the size of these contractions. Noradrenaline is 75-300 times less active in this test. Adult female rat not in oestrus is used. The uterus is even suitable for experiments when kept at 4°C for 24 hour. Excitatory  $\alpha$ -adrenoceptors have been shown to exist in the rat uterus only under certain conditions, such as after oestrogen treatment, during natural oestrus, in late pregnancy, and for five to six days after parturition. The  $\alpha$ -adrenoceptors are temperature sensitive, and the initial excitation phase produced by several sympathomimetic amines is greatly reduced or even abolished by lowering the bath temperature to 25°C.

#### RAT ANOCOCCYGEUS MUSCLE\*

A male rat is killed by a blow on the head and bled. The abdomen is opened in the mid-line, the pelvis split, and the bladder and urethra removed taking care not to damage the ventral band of muscle lying ventral to the colon. The colon is then cut through at the pelvic brim, the pelvic portion pulled forward and the connective tissues cleaned until the anococcygeus muscles come into view. The two anococcygeus muscles in rat arise from the upper coccygeal vertebra behind the terminal colon, pass ventrocaudally to sweep round the lateral side of the colon, and unite in front of the colon a few millimeter from the anus to form a ventral band. The two muscles can be easily dissected out, and the extrinsic nerves that enter the deep surface of each muscle just short of the ventral band can be retained with the muscle for stimulation. The ventral band is cut through and each muscle is mounted in a 100 ml bath.

This smooth muscle preparation is ganglion-free being innervated by long adrenergic neurons, and having muscarinic,  $\alpha$ -adrenergic and 5-hydroxytryptaminergic receptors, all of which produce motor responses. Histamine

\* Gillespie, 1971, 1972.

## Some Standard Isolated Muscle Preparations

is almost ineffective in this preparation. An inhibitory response to field stimulation is obtained after the administration of guanethidine, which is blocked by tetrodotoxin in doses that do not abolish muscle tone or response to noradrenaline, suggesting the inhibitory action is due to nerve stimulation. The nature of the inhibitory transmitter is unknown.

### RAT PHRENIC NERVE—DIAPHRAGM\*

Adult rat is sacrificed and the frontal part of the right thoracic wall is removed after cutting along the right side of the sternum. The mediastinum is cut just above the frontal insertion of the diaphragm taking care not to damage the phrenic nerve. The frontal part of the left thoracic wall is then removed so as to expose the phrenic nerve, and the left lung removed. The left abdominal muscles are cut along the costal margin and the last rib is held with a pair of forceps. Two converging cuts are made through the rib (12 mm apart) towards the tendinous part of the diaphragm (3 mm wide) parallel to its muscle fibres 3 mm on either side of the point where the phrenic nerve enters the diaphragm. The strip is cut out beyond the tendinous part with about 2.5 cm of phrenic nerve attached to it. The fan-shaped preparation is fixed by inserting the rib inside a slit in the platinum electrode. A thread tied around the tendinous end is attached to a light isotonic spring lever. The phrenic nerve is laid on the slit across the two wires and covered with a moist piece of cotton wool. The contractions are recorded on the smoked drum following stimulation of the phrenic nerve and also following direct stimulation of the muscle.

### HAMSTER STOMACH STRIP\*\*

Adult male golden hamster (*Mesocricetus auratus*) 120 to 150 g body weight is given water and food *ad libitum* until killed by decapitation. The stomach is removed, the pouch separated from the main body of the organ and washed in warm Krebs solution. Strips 5 to 7 cm long are then cut by making three to five incisions in the direction of the longitudinal muscle of the pouch. The strip is put up in a 5 ml organ bath.

Hamster stomach strips are useful test preparations for the assay of prostaglandins E and F in nanogram concentration. Compared to the rat stomach, its dose-response curve is steeper, responses are faster and relaxations with small loads on the lever easier. The relative insensitivity of the preparation to 5-hydroxytryptamine and histamine renders it suitable for the assay of prostaglandins in body fluids and tissue extracts. The relative insensitivity of the preparation to prostaglandins  $F_{1\alpha}$ ,  $F_{2\alpha}$  and  $A_1$  compared to  $E_2$  may be taken advantage of in the estimation of mixtures of prostaglandins of different series.

\* Bulbring, 1946    \*\* Ubatuba, 1973



## CAT SPLENIC STRIP

The spleen of the urethanized cat is removed through a lateral abdominal incision and washed in warm Krebs solution. Strips measuring 25 × 5 mm are cut, and put up in an organ bath. An initial tension of 2 to 3 g is applied to the strip and readjusted to 1 to 1.5 g after stabilization for one hour. Cat splenic strips are used for the assay of noradrenaline.

## FROG RECTUS MUSCLE

It is a voluntary muscle preparation producing slow contracture in response to acetylcholine. A pithed frog is laid on its back on a corkboard; the abdominal skin is cut away and the rectus muscle of one side dissected from the pelvic girdle up to the pectoral girdle. The muscle is then pinned to the cork so as to keep it stretched to its normal length soaked in frog saline. A thread is sewn through each end, and the muscle is then fixed in the bath, while the thread from the other end is fixed to a simple or Gimbal lever.

The preparation is suitable for the assay of acetylcholine as well as for curare-like substances.

Table 17.1 summarizes the experimental details of some common isolated preparations.

Table 17.1  
Summary of experimental details for some common isolated preparations

Preparation	Salt solution	Temp (°C)	Gas	Lever	Tension (g)	Magnification
Guinea-pig ileum	Tyrode	35-37	O <sub>2</sub> or Air	Isotonic frontal	0.5-1	5-10
	Ringer	32	Air	"	0.5	"
ileum (coaxial)	Krebs	37	Carbogen*	"	0.5-1	"
	"	37	"	"	0.2-0.5	12-20
vas deferens	Tyrode	32-34	Air	"	0.5-1	4-8
	Ringer	37	"	"	"	"
	McEwen	29	Carbogen	"	"	"
	Krebs	31	"	"	"	"
seminal vesicle	McEwen	32	"	"	0.2-0.5	8
taenia caeci	"	35	"	"	1.5	4
	Ringer	37	"	"	"	"
atria	Krebs	30	"	Starling heart	0.5-1	7-10
	McEwen	32-37	O <sub>2</sub>	"	"	"
	Ringer (double glucose)	29-30	"	"	"	"

\* .5% CO<sub>2</sub> in O<sub>2</sub>

## Some Standard Isolated Muscle Preparations

Table 17.1 (Contd.)

Preparation	Salt solution	Temp (°C)	Gas	Lever	Tension (g)	Magnification
Rabbit duodenum	McEwen	37	Carbogen	Isotonic frontal	4	9
duodenum or ileum.	Tyrode	34-38	Air	"	1-3	5
ileum or jejunum	Ringer	"	"	"	"	"
jejunum	Krebs	37	Carbogen	"	"	"
innervated jejunum (Finkelman)	Tyrode	"	"	"	1	"
aorta	Krebs (double glucose)	"	"	"	2-4	7-10
	Krebs (half bicarbonate)	37	O <sub>2</sub>	"	4	"
	McEwen	37	O <sub>2</sub> or Carbogen	"	4	4 or 9
Rabbit heart	Ringer (Langendorff)	37 (double glucose)	O <sub>2</sub>	Starling	4 heart	4 or 9
atria	Krebs	" (double glucose & half bicarbonate)	"	"	0.5-1.5	"
	McEwen	29	Carbogen	Isometric		
Rat ileum or duodenum	de Jalon	30-31	O <sub>2</sub> or Carbogen	Isotonic Carbogen	0.5 frontal	15
ileum	Tyrode	37	O <sub>2</sub>	"	0.25	7
ascending colon	Krebs	37	Carbogen	"	2	4
descending colon	de Jalon	25	O <sub>2</sub> or Carbogen	"	"	"
stomach (fundus)	Tyrode	37	O <sub>2</sub>	"	1	5-16
	de Jalon or Krebs	"	Carbogen	"	"	"
anococcygeus	Krebs	36 or 37	"	"	0.2-1	10
uterus	de Jalon	25-36	O <sub>2</sub> or Carbogen	"	0.5-1	4
	Krebs	37	Carbogen	"	4	"
	McEwen	34	O <sub>2</sub>	"	"	"
	Locke	37	Carbogen	"	"	10
vas deferens	Krebs	37	Carbogen	Isotonic	0.5-1 frontal	6
	Ringer	"	Air	"	0.5	6-8

Table 17.1 (Contd.)

Preparation	Salt solution	Temp (°C)	Gas	Lever	Tension (g)	Magnification
phrenic nerve-	Tyrode	32	Carbogen	"	1	6
	McEwen	32 or 37	"	"	0.35	"
Mouse	Tyrode	31 or 37	O <sub>2</sub> or	Spring	loaded or simple	8-18
ileum	diaphragm	(double glucose or Krebs)		Carbogen		
vas deferens	Ringer	37	Air	Isotonic	0.2	6-8
Frog	"	"	"	"	frontal	"
	Krebs	37	Carbogen	"	0.5-1	0.1-1
rectus	Hukovic	32	"	"	0.3	10-15
Cat	Frog Ringer	Room	O <sub>2</sub> or Air	Simple or gimbal	0.5-1	10
6-10 frontal	splenic strip	Krebs	37	Carbogen	Isotonic	1-1.5
		McEwen	38	"	"	0.5
10 Hamster stomach strip	Krebs (Single glucose)	37	"	"	0.5-2	"

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# Quantitative Study of Agonists on Isolated Preparations

The estimation of potency of an unknown concentration of an agonist (*test*) is carried out by comparing its activity with the same agonist of known strength (*standard*), and finding out the equiactive dose. This is what is called *analytical dilution assay*, because the assay involves the determination of the factor by which the *test* agonist is diluted or concentrated compared with the *standard* agonist. The nature of the test and of the standard being qualitatively the same, this type of assay gives almost identical results whatever may be the test organ, the species or the method. This is in contrast to *comparative assay* where the test compound is chemically different from the standard though having similar activity. In such an assay, testing on different tissues may give different results because of difference in sensitivity to two different substances (Schild, 1950).

## MATCHING OR BRACKETING ASSAY

The simplest type of graded assay is one for which no statistics is required, and in which the standard and unknown are matched by a trial and error process until they produce equal effects. It has one great advantage that it does not depend on the assumption of a dose-response relationship, but has a number of disadvantages as follows: (a) it is inefficient since the preliminary effects are not utilized in the final assessment, (b) the match is purely subjective, (c) experimental error cannot be determined from the assay itself, and (d) it gives no indication of the parallelism of the dose-response curves of standard and unknown, that is of the qualitative differences, since the effects are matched at only one dose level.

The working dose of the standard is first determined in the sensitive part of the dose-response curve. In practice it is not necessary to determine the whole range of the dose-response curve but to select a dose that will produce approximately 50 per cent of the maximal contraction. The simplest way of doing this is to give sufficiently large dose of the drug so as to get a maximal contraction, and to confirm by giving still larger dose after washing, showing no further increase in the response. The lever should be so adjusted that the contraction height is of sufficient length that is recorded completely on the smoked surface. The tissue is

then washed several times till the lever comes back to the original base line, and a dose is selected which produces almost half the maximal contraction.

Usually the dose of the standard is kept constant throughout the experiment to have some idea about the change in the sensitivity of the tissue with time. The standard is added to the bath at a fixed interval alternating with the test, so that each contraction produced by the *test* is bracketed (flanked) by contractions produced by fixed doses of the *standard*. At first a particular volume of the *standard* solution is given producing sufficient response, followed by the same volume of the *test* solution, again followed by the same volume of *standard* solution. The doses of the *test* are then increased or decreased accordingly to obtain contractions just larger and smaller than the average of those of the *standard* flanked on either side. If a matching is obtained right at the outset, it could have been fortuitous due to change in the sensitivity of the tissue rather than due to the dose, and is not acceptable. Close bracketing is liable to give more accurate approximation, from which an equivalent dose of the *test* is tried. The matching becomes perfect if almost identical responses are obtained by giving two to three times the equivalent doses of the *standard* and of the *test*.

*Example:* An unknown solution of histamine (T) was estimated by comparison with a known solution (S) on isolated guinea pig ileum as illustrated in Fig. 18.1.

The effects of equal doses of the S and T were observed first, and then the effects of T and S were bracketed as closely as possible. Thus, 0.2 ml T produced bigger effect than 0.2 ml S; 0.15 ml T still produced bigger, while both 0.1 ml and 0.12 ml produced smaller effects than 0.2 ml S; 0.14 ml T produced same effect as 0.2 ml S; finally, two and a half times the equivalent doses of both T and S produced almost identical effects.

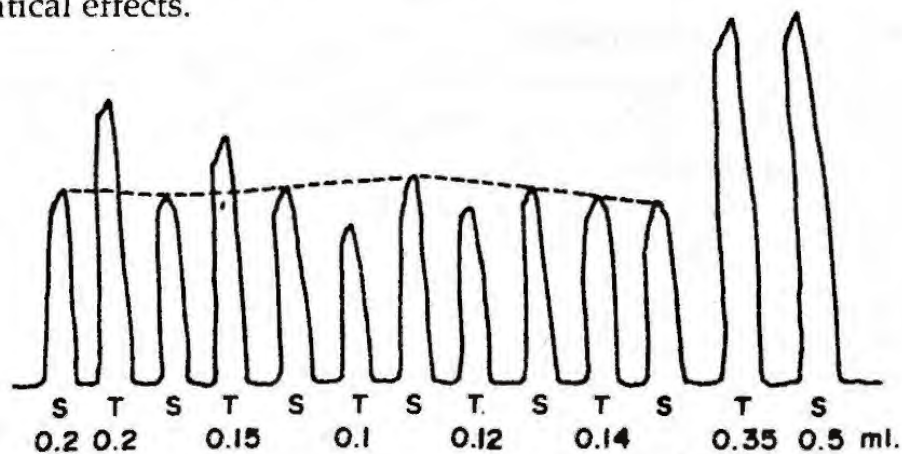


Fig. 18.1. Matching assay of an unknown solution of histamine on guinea pig ileum. For details see text.

Since,  $0.2 \text{ ml S} = 0.14 \text{ ml T}$ ,  
 $1 \text{ ml S} = 0.70 \text{ ml T}$

Now, 1 ml S contains 10 mg of histamine

Hence, 1 ml T is estimated to contain  $10/0.7$ , i.e. 14.3 mg of histamine

But T actually contained 15 mg/ml of histamine

The error of the assay =  $[(15-14.3)/15] \times 100 = 4.7\%$

Sometimes, a geometric mean (G.M.) rather than arithmetic mean (A.M.) of the close-bracketed values gives a better estimate.

### DOSE-RESPONSE CURVES

For determination of the dose-response relationship, graded effects are obtained by administering varying concentrations (or doses) of the drug by the *intermittent dose method*, that is, washing out the preceding dose before adding the next. At least four or five varying doses are used to cover the range between the smallest and the largest (maximal) responses. *Maximal response* is that which does not increase further with increase in the dose. For the convenience of plotting the dose-response curve, the doses are increased in geometric progression (logarithmic intervals), because the response is roughly linearly related to the log dose. In order to do this, each dose is obtained by multiplying the preceding dose by some constant factor, termed the *common ratio* (e.g. by 2,  $\sqrt{2}$ , 0.5, etc.). It is better to administer the doses in a random fashion rather than to give in ascending or descending order. In order to obtain a fairly smooth dose-response curve at least four or five responses should be obtained for each dose, and the mean effect plotted against each dose.

The logarithms of doses (independent variables) are first plotted along the abscissa or X-axis (horizontal scale). If the doses are in geometric progression (log intervals) they can be plotted directly at equidistant intervals; while if they are in arithmetic progression, each dose is converted into log value, which is then plotted along the abscissa. Alternatively, the original doses are plotted directly on a logarithmic paper. The responses (dependent variables) are measured for each dose, and the mean responses (or the percentage of maximal responses) are then plotted along the ordinate or Y-axis (vertical scale) against the corresponding doses.

The logarithmic transformation of dosage has the following advantages:

- a) Results can be plotted when the doses vary even over a 1000-fold range which otherwise is not possible.
- b) Dose-response curve becomes linear particularly in the middle part.
- c) The error is generally normally distributed and independent of the dose.
- d) Has certain mathematical advantages when the two dose-effect curves are compared.

For example, the horizontal distance between the two parallel lines is a measure of the potency ratio of the two drugs.

A log dose-response curve has a characteristic sigmoid or S-shape with linear portion in the middle that can sometimes be further extended by transformation of responses (parameters) into corresponding probits, squares, reciprocals, etc. (metameters). The lower and the upper parts of the curve are relatively flat approaching respectively zero and maximum asymptotically, while the middle linear portion is relatively steep. As can be seen from the Fig. 18.2 the middle

linear portion is the most sensitive part of the curve and the working doses are therefore selected around this region for any quantitative assay.

The dose-response curve is characterized by three parameters:

(i) *Maximal or ceiling effect.* Commonly referred to as  $E_{max}$  or *efficacy*, which should not be confused with the term potency. Potency may vary between similar drugs, while the efficacy remains the same. For instance, hydrochlorothiazide, which is about 20 times more potent than chlorothiazide as a diuretic, has a similar efficacy. Furosemide, on the other hand, is not only 10 times more potent than chlorothiazide but also has a higher ceiling effect, that is, efficacy. Different efficacy suggests different mechanisms of drug action.

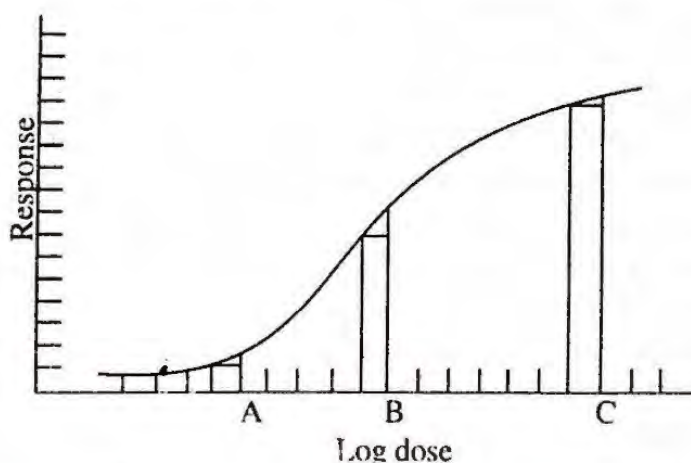


Fig. 18.2. Sigmoid dose-response curve showing sensitive (steep) middle portion and relatively insensitive (flat) lower and upper portions. The same increase in dose produces greater increase in response at B than at A or C.

(ii) *Position of the curve.* In relation to the abscissa it gives an idea about the affinity or potency of the drug. For example, when the curve is more towards the left, it is more potent, and *vice versa*.

The potency of a substance depends on a number of factors, such as, its accessibility to the tissue, number of receptors combined with it, its affinity for the receptors, and its efficacy.

The potency of a drug may be expressed as ED50 or EC50 (median effective dose or concentration), that is a dose or concentration producing 50 per cent of the maximal response. Lower the value, more potent is the drug. All horizontal shifts of dose-response curves are measured at the level of ED50 or EC50. It is preferable to use the term ED50 for *in vivo*, and EC50 for *in vitro* studies.

Another way of expressing the potency, particularly when the values are rather inconvenient numbers, such as molar concentration, is by the  $pD_2$  value (analogous to pH), which is the mean *negative logarithm of molar concentration* producing 50 per cent of maximal response (Ariens and van Rossum, 1957). Higher the  $pD_2$  value, higher is the potency.

*Calculation of  $pD_2$  value.* The percentage of the maximal response produced by each dose is plotted against the log molar concentration of the drug, and a concentration-action curve is obtained. The 50 per cent point of the curve is projected on the concentration axis (abscissa), and the value is read out. In case the projection falls between the points indicating the whole division of ten, the distance to the nearest point at the left is measured. Suppose, the nearest point at the left indicates  $10^{-6}$  and the projection is on the 4th division, then the sought concentration is  $10^{-6.4}$  or  $10^{-5.6}$ . The  $pD_2$  value therefore is 5.6 (Fig. 18.3). Ariens and van Rossum (1957), however, employed 30 divisions along the concentration axis in their studies.

(iii) *Slope of the curve.* Also known as the *regression coefficient (b)*, which determines the error or reliability (precision) of an assay. The value of a biological method depends on the rate at which the effect increases in relation to the increase in the dose. Steeper the slope (i.e. higher the  $b$  value), more precise is the assay, and *vice versa*. In other words, with steeper slope the discrimination between doses is good, that is, small differences in doses produce large differences in response. A simple method of finding out the slope of a dose-response curve is to calculate the ratio of the concentration of responses producing 80% and 20% maximal effects (Stephenson, 1956), or as the quotient "60 divided by (log EC80-log EC20)" (Langer and Trendelenburgh, 1969). It may be mentioned here, that doubling the dose of a drug does not necessarily produce doubling of an effect. Thus, the magnitude of the effects produced by the same dose of two drugs cannot be the basis of comparison. For instance, if one drug produces a response twice the size of that produced by the same dose of another drug, the first drug is not necessarily twice as potent as the second drug. Determination of doses of the two drugs producing almost equal effects is usually the basis of comparison.

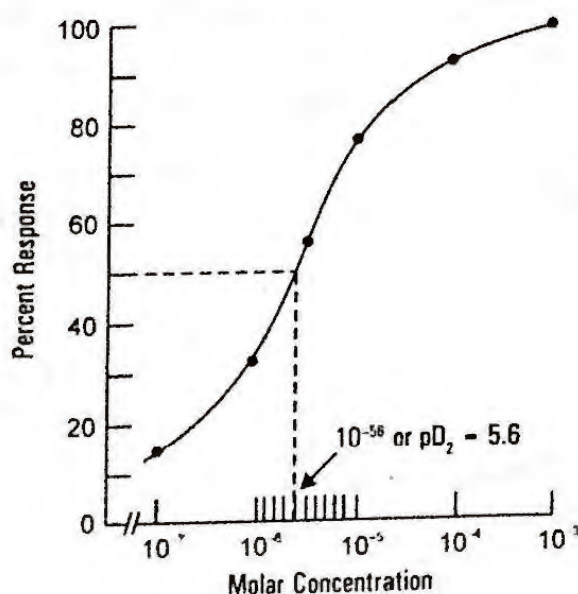


Fig. 18.3. Graphical calculation of  $pD_2$  value. For details see text.



LOG DOSE-RESPONSE LINE OR REGRESSION LINE

The response is directly proportional to the log dose, that is, linear within limits of 20 to 80 percent of the maximum. The experimentally observed points on a graph are scattered about the line, which is usually drawn to the eye as the best fitting line. The slope and the position of the line, however, are bound to vary due to the subjective bias of the experimenter. There is, however, a mathematical procedure, the method of least square, that gives the best fitting line to the experimental data. The principle of least square states that, a line of best fit to a series of values is a line, the sum of the squares of the deviations of individual observations about which will be minimum. In addition to finding the best fitting line, its distance above the base line  $a$ , and its slope  $b$  have to be defined. Standard statistical theory states, that the line best representing these relationships has the following linear regression equation:

$$Y = a + bX$$

where,  $a$  is the distance above the base line at which the regression line cuts the Y-axis, and  $b$  (regression coefficient) is the degree of the slope, or the inclination of the regression line to the X-axis. The regression coefficient is usually positive indicating that the inclination of the regression line is upwards towards the right. When it is negative, the inclination is downwards towards the right.

The formulas for the regression coefficient  $b$  and for  $a$  are as follows :

$$b = S_{xy} / S_x^2, \text{ where } x = (X - \bar{X}), \text{ and } y = (Y - \bar{Y})$$

$$a = \bar{y} - b\bar{x}, \text{ where } \bar{x} \text{ and } \bar{y} \text{ are the mean values of X and Y respectively.}$$

*Example:* Calculation of the regression line of the sleeping time in three groups of mice (5 in each group) following three different volumes of ether administered for a fixed time is presented below (Ghosh *et al.* 1964):

Total number of mice = $n$	Log vol. ether (ml) = $X$	Sleeping time (sec) = $Y$
$n = 15$	$SX = 34.7$	$\bar{x} = 2.31$
	$SY = 499$	$\bar{y} = 33.3$
	$SX^2 = 80.39$	$(SX)^2 = 1204.1$
	$SXY = 1163.8$	$(SX)(SY) = 17315.3$

$$\begin{aligned} S_x^2 &= SX^2 - (SX)^2/n \\ &= 80.39 - 80.27 \\ &= 0.12 \end{aligned}$$

$$\begin{aligned} S_{xy} &= SXY - (SX)(SY)/n \\ &= 1163.8 - 1154.4 \\ &= 9.4 \end{aligned}$$

$$\begin{aligned} b &= S_{xy}/S_x^2 \\ &= 9.4/0.12 \\ &= 78.3 \end{aligned}$$

$$\begin{aligned} a &= \bar{y} - b\bar{x} \\ &= 33.3 - (78.3 \times 2.31) \\ &= -147.6 \end{aligned}$$

The regression equation of Y on X is

$$\begin{aligned} Y &= a + bX \\ &= -147.6 + 78.3 X \end{aligned}$$

## Quantitative Study of Agonists on Isolated Preparations

Taking three values of  $X$  and substituting these in the equation, we get:

$$X_1 = 2.20, Y_1 = -147.6 + (78.3 \times 2.20) = 24.7$$

$$X_2 = 2.32, Y_2 = -147.6 + (78.3 \times 2.32) = 34.1$$

$$X_3 = 2.42, Y_3 = -147.6 + (78.3 \times 2.42) = 41.9$$

A regression line is fitted as per the equation through these points (Fig.18.4):

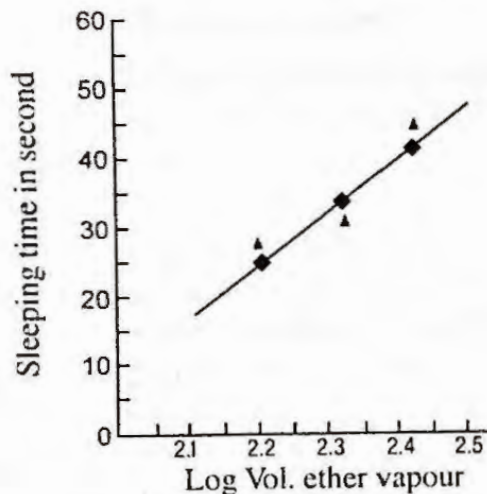


Fig. 18.4. Regression line fitted by the method of least square. For details see text.

### EVALUATION OF AGONIST

From the dose-response curve three parameters can be determined directly:

(i) *Intrinsic activity (a)*: expressed as the ratio between the maximum effect of the drug under study ( $E_{maxT}$ ) and that of the reference drug ( $E_{maxS}$ ) of a homologous series:

$$a = E_{maxT} / E_{maxS}$$

(ii) *Affinity*: Expressed as  $EC_{50}$  or  $pD_2$  value. Conversion of  $EC_{50}$  to  $pD_2$ :

If  $EC_{50}$  is expressed as  $(m \times 10^{-n})$ ,

then  $pD_2 = n - \log m$ ,

where  $m$  is molar concentration

Example:  $EC_{50} = 9.5 \times 10^{-6}$

$$pD_2 = n - \log m$$

$$= 6 - \log 9.5$$

$$= 6 - 0.98$$

$$= 5.02$$

(iii) *Slope of the curve*: Expressed as regression coefficient ( $b$ ).

The responses to agonists are generally more linearly related to the geometric rather than to the arithmetic increments in dose. Parametric statistical procedures, such as Student's  $t$  test assume that the mean used in the calculation is based on its

normal distribution. The log values of equieffective doses (EC<sub>50</sub>) of agonists are normally distributed. Therefore, the mean of equieffective doses, and the test of significance should be based on mean log or antilog, i.e. on the geometric mean (G.M.) and not on the arithmetic mean.

G.M.s and their 95% confidence limits are calculated as described in Chapter 34.

### ASSAY DESIGN

(2 and 1) *dose assay*. Responses are obtained with two doses of the standard in the linear range of the dose-response curve, and a dose of the test is found that produces a response between these two doses. The dose of the standard producing exactly the same response as the test is found out by interpolation from the log dose-response curve of the standard. In 95 per cent of cases, the assumption regarding a linear dose-response relationship and parallelism of the dose-response lines for unknown and standard is probably true.

(2 and 2) *dose assay*. A satisfactory method for general use is a (2+2) dose assay, in which error of the method can be calculated from the internal evidence. It is preferable also from the point of view of reasonable accuracy and economy of time and of the animals. The test consists of three parts (Schild, 1942):

- i) Selection of two doses of standard near the steepest part of the curve, that is, in a region where a given increase in the dose produces the maximum increase in the response. This is obtained by increasing or reducing the dose by say, a multiple of two until the range of greatest sensitivity is found, and responses one near 30 per cent, and the other near 70 per cent of the maximal are selected. These doses may be in the ratio of 1:2 or 3:2 or in some other ratio. It is obvious that the larger the ratio of 'high' to 'low' dose, the less precise will be the estimate.
- ii) A preliminary estimate of the strength of the unknown solution is made by a *matching assay*. A dilution of the unknown or the standard is then made, so that equal volumes of unknown and standard contain approximately the same activity by this rough estimation.
- iii) The main part of the test consists in adding the two selected doses of standard, and the two selected doses of unknown in the same ratio to the bath one after another at regular intervals. The addition of these four doses are then repeated five or six times, varying the order of doses in each group randomly, or in a latin square design.

The test is valid provided the following conditions are fulfilled: (a) a linear log dose-response exists throughout the range of doses used, (b) effects of any one dose are normally distributed, (c) standard deviation of the effect is independent of the effect itself, (d) a small value of  $s/b$  ( $\lambda$ ) which is a good measure of precision, and (e) allocation of the order of doses in different groups is made by some random process. It is generally sufficient to assume the first three

conditions to be generally true, if previous experiments have made it likely that condition (a) will be fulfilled.

When the response is linearly related to log dose, and the two regression lines are parallel, the horizontal distance  $M$  between the regression lines is the logarithm of the relative potency. The antilog of  $M$  gives the potency ratio. The precision of comparison is greater, steeper the regression lines (i.e. higher the values of  $b$ ). The ratio of the variance to regression coefficient ( $s/b=\lambda$ ) is known as the *index of precision*. This index is independent of the units in which doses are measured, and also of the experimental design, viz, arrangement of doses, number of animals used, etc. The lower the index, the more precise is the assay. Table 18.1 gives some idea of the precision of some common bioassays. The lambda value ( $\lambda$ ) of 0.05 and below is excellent, 0.1 to 0.2 is acceptable, and 0.5 and above not acceptable.

**Table 18.1**  
Indices of precision  
Comparisons on the same animal or tissue in acute experiments (2 + 2) dose assays†

Drug	Animal	Response/Tissue	$\lambda = s/b$
Adrenaline	Dog	Blood pressure	0.2
			0.038
	Rat	Colon	0.033
		Uterus	0.13
Posterior pituitary	Rat	Uterus	0.062
			0.057
	Guinea pig	Uterus	0.2
	Chicken	Blood pressure	0.043
Histamine	Guinea pig	Ileum	0.033
Methantheline/ Atropine*	Rats-2	Gastric acid secretion	0.09
	Rats-4		0.25

† Modified after Gaddum, 1953.

\* Comparative assay in two and four rats (Ghosh, 1958).

## CUMULATIVE DOSE-RESPONSE CURVE

The cumulative dose-response curves may be obtained by increasing the concentration of the drug in the bath fluid step by step without washing out the preceding doses, that is, by the *cumulative dose method* (van Rossum and van den Brink, 1962). This technique is simple and requires much less time than the conventional technique where each dose is added after washing out the preceding dose. The dose-response curves obtained by either method are virtually the same.

This technique, however, cannot be adopted if the drug is unstable or fading occurs, i.e. the response to a dose first reaches its peak and then decreases to a steady level without washing. Nonspecific desensitization can be expected to be greater by the time the top of the cumulative dose-response curve is reached than

if the maximal dose were given to a fresh preparation by the intermittent conventional method. The organ bath should not be too small and repeated addition of drug solutions without washing should not affect the volume by more than five per cent (e.g. for 10 ml bath the total volume of drug solution added is 0.5 ml or less). After stabilization of the tissue, the agonist is added into the bath in cumulative amounts (usually in molar concentration) every time the equilibrium is reached following a dose. The sequence 1, +2, +7, +20, +70, +200, etc. is used so that the drug concentration in the bath increases stepwise in the series 1, 3, 10, 30, 100, 300, etc. at 0.5 log unit intervals. Along with the increase in concentration, the contraction of the tissue increases step by step without relaxation. If the next concentration does not cause a further increase in contraction, it is assumed that the maximum effect for that particular drug is reached, and the drug is then washed out (see Chapter 19, Fig. 19.2). Cumulative dose-response curves of the standard are made alternately with those of the test compound. The effect of each cumulative dose is expressed as percentage of the maximum height of the preceding curve of the standard, and plotted against log dose. A difference in the position of the curves on the abscissa indicates a difference in potency.

The relative potency can also be calculated from the following formula:

Relative potency (affinity ratio) =  $100 \times \text{antilog mean (negative log EC}_{50} \text{ of test - negative log EC}_{50} \text{ of standard)}$ , where standard is assigned a value of 100 (Table 18.2 and 18.3).

Table 18.2  
Potency of terbutaline compared with isoprenaline on guinea pig isolated trachea and atria\*

Negative log EC <sub>50</sub> ± s.e. (Molar conc.) (pD <sub>50</sub> )					
	Trachea		Atria		
	Terbutaline	Isoprenaline	Terbutaline	Isoprenaline	
	7.09 ± 0.05	7.60 ± 0.05	5.43 ± 0.08	8.87 ± 0.06	

\* Data from O'Donnell and Wanstall, 1974

Values are mean of five observations

Table 18.3  
Potency ratio of terbutaline and isoprenaline

	Trachea	Atria
Difference* between pD <sub>50</sub> of terbutaline and isoprenaline	- 0.51	- 3.44
Relative potency of terbutaline (isoprenaline = 100)	30.9	0.036

\* Differences are derived from Table 18.2

## Quantitative Study of Agonists on Isolated Preparations

Table 18.4  
Selectivity ratio of terbutaline and isoprenaline

	Terbutaline	Isoprenaline
Difference* between $pD_{25}$ for trachea and atria	1.66	- 1.27
Selectivity ratio trachea : atria (antilog difference)	45.7	0.054

\* Differences are derived from Table 18.2

**Selectivity.** The selectivity of a compound for one tissue or the other can be determined by estimating its EC50 value on both the tissues being examined. The *selectivity ratio* is then calculated from the following formula:

*Selectivity ratio* (tissue<sub>1</sub> / tissue<sub>2</sub>) = antilog mean (negative log EC50 on tissue<sub>1</sub> - negative log EC50 on tissue<sub>2</sub>) (Table 18.2 and 18.4).

*Relative potency (affinity ratio)* values may also be computed for selectivity ratio. This is a more satisfactory method, since it eliminates the variability associated with sensitivity differences between the experimental animals or the tissues.

A value much greater than or less than one indicates selectivity to one tissue or the other, while a value nearing unity suggests nonselectivity.

**Potentiation.** Dose-response curves are obtained before and after treatment with a potentiating agent and the value for potentiation is calculated as per the formula below:

*Potentiation* = antilog mean (negative log EC50 after treatment - negative log EC50 before treatment).

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# Quantitative Study of Antagonists on Isolated Preparations

An antagonist (say, an antispasmodic) can be assayed against either a spontaneous or a spasmogen-induced contraction. Antagonism by way of relaxation of the spontaneously increased tone of guinea pig trachea is obtained by adrenaline, papaverine as well as aminophylline, while that of rabbit duodenum by adrenaline alone. Atropine and related drugs, and antihistamines, however, do not produce any relaxation of these preparations as such, but they effectively antagonize the contractions induced by the specific spasmogens.

It is important to realize that the full effect of an antagonist may take many minutes or even hours to develop, unlike stimulant drugs, where the effect reaches its peak fairly rapidly. Speed of onset of antagonism is directly proportional to the concentration; hence it is slower with more potent drug given in smaller concentration than with less potent drug given in higher concentration.

The antagonisms of the drug-induced contractions are of two types:

1. *Preventive* — where an antagonist added first into the bath prevents or inhibits the effect of an agonist added subsequently. Antagonism of histamine is satisfactorily tested on guinea pig ileum, of acetylcholine and barium on rabbit ileum, and of oxytocics on guinea pig or rat uterus. Aminophylline, which is ineffective in antagonizing histamine action on the intestine, is quite active in antagonizing its action on the guinea pig trachea.
2. *Curative* — where a spasm is first induced usually in guinea pig trachea, or in rabbit ileum with any of the standard agonists, like histamine, acetylcholine, barium, etc.; the antagonist is then added into the bath to bring about relaxation.

## DETERMINATION OF IC<sub>50</sub> VALUE

One of the simplest ways of expressing an antagonist quantitatively is to find out the concentration that will produce 50 per cent inhibition (IC<sub>50</sub>) of the effect of an agonist.

Two or three equal submaximal contractions are first obtained with a spasmogen. One minute earlier to the subsequent dose of the spasmogen, a dose

of the antagonist is added sufficient to produce, say around 70 to 80 percent contraction produced by the dose of spasmogen alone. After washing out the antagonist, the spasmogen is added repeatedly with washings, till there is almost complete recovery of the effect of the spasmogen. A dose of antagonist is now added so as to produce around 20 to 30 per cent contraction. The whole procedure is repeated at least four or five times for each dose, both for the standard as well as for test compound. The mean percentage inhibition by each dose is plotted against the log dose. The 50 per cent inhibitory dose (IC 50) is found out by interpolation. The relative potency of a standard and of a test compound can thus be compared. It is important that both the standard and the test compound are studied simultaneously on the same preparation for reliable estimation.

IC50 value can also be estimated for the *cumulative* type of antagonism in the following way (Ludueno and Lands, 1954). A contracture of an isolated rabbit ileum is first obtained with acetylcholine. After two minutes, the antagonist is added to the bath and the resulting reduction of acetylcholine-induced contracture occurring during the subsequent two minutes is expressed as percentage of the total contracture. The drugs are then washed out, and the muscle strip washed twice with fresh bathing fluid, and again five minutes later. Before testing the next dose of antagonist, typical spastic contracture should be obtained with acetylcholine again. The effect of four or five doses at logarithmic intervals is determined. The mean percentage reduction in contracture of five to six determinations for each dose is plotted against the log dose. The IC50 is estimated from the log dose-response curve and compared with that of the standard.

### DETERMINATION OF DOSE RATIO

The effect of antagonism can be measured in terms of the *dose ratio* by finding out the equiactive doses of agonist in the presence and in the absence of the competitive antagonist:

$Dose\ ratio = EC_{50}\ after\ antagonist / EC_{50}\ before\ antagonist.$

The *dose ratio* is the factor by which the concentration of the agonist must be multiplied to produce a given response in the presence of the antagonist. Higher the dose-ratio, more specific is the antagonist. The dose-ratio generally increases with the time of exposure; hence it is desirable to adopt a standard time of exposure (say, 14 or 60 min) in order to avoid the errors inherent in very long experiments.

### DETERMINATION OF PERCENTAGE INHIBITION

Antagonism can also be expressed in terms of percentage inhibition, which is calculated as follows:

$Percentage\ inhibition = 100 - \left[ \frac{\text{height of contraction (or size of any other response) after exposure to antagonist}}{\text{that before exposure to antagonist}} \times 100 \right]$



RELATIVE POTENCY FROM DOSE-RESPONSE CURVES

The log dose-response curves are plotted for the standard and for the test antagonist using four or five varying doses against a constant dose of the agonist. The two curves are usually parallel, and the horizontal distance between the two gives an estimate of the *potency ratio*.

As with the agonist, a (2 and 2) dose assay may also be carried out with antagonists. Two concentrations of the antagonist (each for standard and for test), one producing less than 50 percent and the other producing more than 50 percent inhibition of the effect of a constant dose of the agonist are administered two minutes before the constant dose of the agonist. Between each administration of the antagonist, several doses of the agonist are repeated until there is a complete recovery of the response.

*Mathematical calculation of the potency:* The calculation depends on the assumption that the response bears a linear relation to log dose.

*Example:* Finding out the relative activity of atropine and methantheline on methacholine induced gastric acid secretion in the rat by (2+2) dose assay (Ghosh,1958). Results are presented in Table 19.1

Table 19.1  
Results of (2+2) dose assay of atropine and methantheline on methacholine induced gastric secretion in rat

Drug Mean	Dose (µg)	Maximum deflexion of pH in different groups				Total
Atropine (S <sub>1</sub> ) 1.52	1.0	1.4	0.9	1.9	1.9	6.1
Methantheline (T <sub>1</sub> ) 1.46	0.1	0.9	0.8	2.35	1.8	5.85
Atropine (S <sub>2</sub> ) 0.52	3.0	0.3	0.25	1.1	0.45	2.1
Methantheline (T <sub>2</sub> ) 0.44	0.3	0.3	0.2	0.55	0.7	1.75
S <sub>1</sub> = 1.52	S <sub>2</sub> = 0.52	T <sub>1</sub> = 1.46		T <sub>2</sub> = 0.44		

Log ratio of dose (l) = log 3 = 0.48  
 Dose difference (E) =  $\frac{1}{2}(T_2 - T_1 + S_2 - S_1) = \frac{1}{2}(0.44 - 1.46 + 0.52 - 1.52) = -1.01$   
 Preparation difference (F) =  $\frac{1}{2}(T_1 + T_2 - S_1 - S_2) = \frac{1}{2}(1.46 + 0.44 - 1.52 - 0.52) = -0.07$   
 Slope (b) = E/l =  $-1.01/0.48 = -2.1$   
 Log ratio potency T/S or M = F/b =  $-0.07/-2.1 = 0.033$   
 Potency ratio (antilog of M) = 1.08

Since methantheline produced slightly more inhibition than atropine, and its dose was 10 times less than that of atropine :

$\frac{\text{Methantheline } 0.3 \mu\text{g}}{\text{Atropine } 3.0 \mu\text{g}} = 1.08$

Therefore, methantheline =  $\frac{1.08 \times 3.0}{0.3}$ , i.e 10.8 × atropine

## Quantitative Study of Antagonists on Isolated Preparations

In other words, methantheline is about 11 times more potent than atropine in reducing methacholine induced gastric acid secretion in the anaesthetized rat.

Almost similar result is obtained graphically when the response is plotted against the log dose and the horizontal distance between the two regression lines is measured (Fig. 19.1). The potency ratio between atropine and methantheline appears to be  $M = 1.03$  whose antilog is 10.7, which is very close to the value 10.8 obtained mathematically

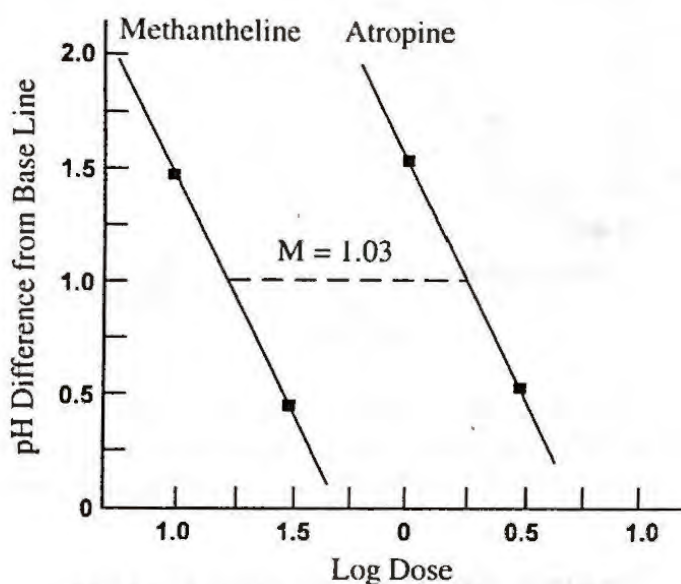


Fig. 19.1. Graphical method of finding out the potency. Note the negative regression lines.

### CUMULATIVE DOSE-RESPONSE CURVES STUDY OF ANTAGONISM

Cumulative dose-response curves with an agonist are recorded alternately in the absence and in the presence of different concentrations of an antagonist (Fig. 19.2).

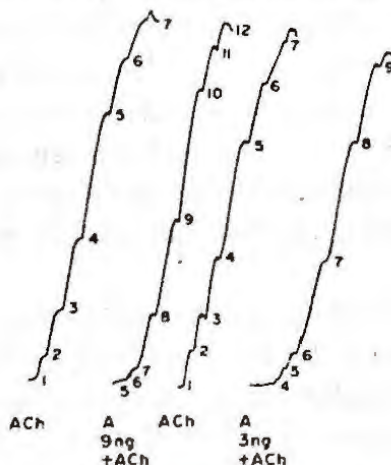


Fig. 19.2. Cumulative dose-response curves of acetylcholine (ACh) on mouse ileum in absence and in presence of atropine (A)

From these, dose-response curves are plotted as percentage response against the log dose of agonist for different concentrations of the antagonist (Fig. 19.3)

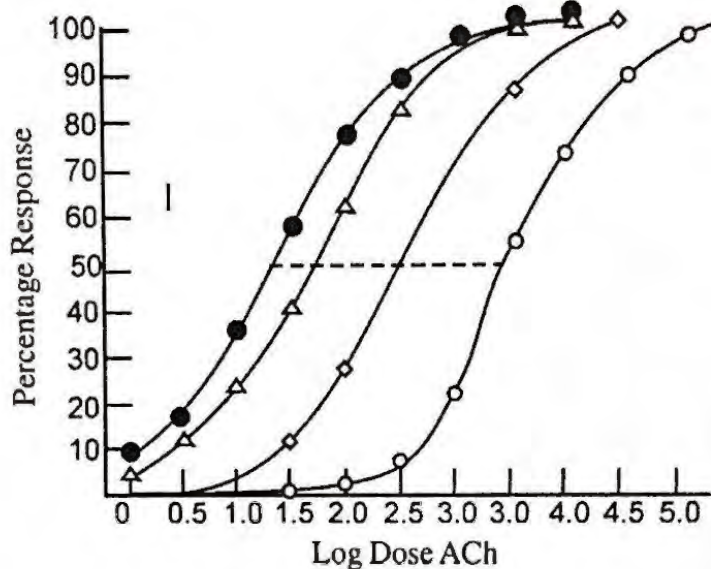


Fig. 19.3. Log dose response curves of acetylcholine (ACh) in absence and in presence of atropine. These are plotted from cumulative dose-response curves of ACh on mouse ileum.  
 ●—● ACh alone; in presence of atropine Δ—Δ 1 ng; □—□ 3 ng; ○—○ 9 ng.

### TYPES OF DRUG ANTAGONISM

#### Competitive Antagonism

An antagonist is said to be *competitive* if it competes with the agonist for the same binding site on the receptor.

*Reversible (surmountable or equilibrium) competitive antagonism.* When the bond formed between the antagonist and the receptor is loose, the receptor-antagonist complex can dissociate thereby producing classical reversible competitive antagonism. The antagonism increases as the concentration of the antagonist is increased. Conversely, the antagonism can be overcome (*surmounted*), if the concentration of the agonist is increased. The concentration-response curves of the agonist are parallel and shifted to the right more and more with increasing concentrations of the antagonist, but the maximal response remains the same (Fig. 19.4).

*Irreversible (insurmountable or nonequilibrium) competitive antagonism.* The bond formed between antagonist and the receptor is of the covalent type. As the dose of the antagonist is increased the slope as well as the maxima of the agonist curve are progressively depressed. With sufficiently high dose of antagonist no amount of agonist will produce a response. Dibenamine and phenoxybenzamine are antagonists belonging to this category (Fig. 19.5).

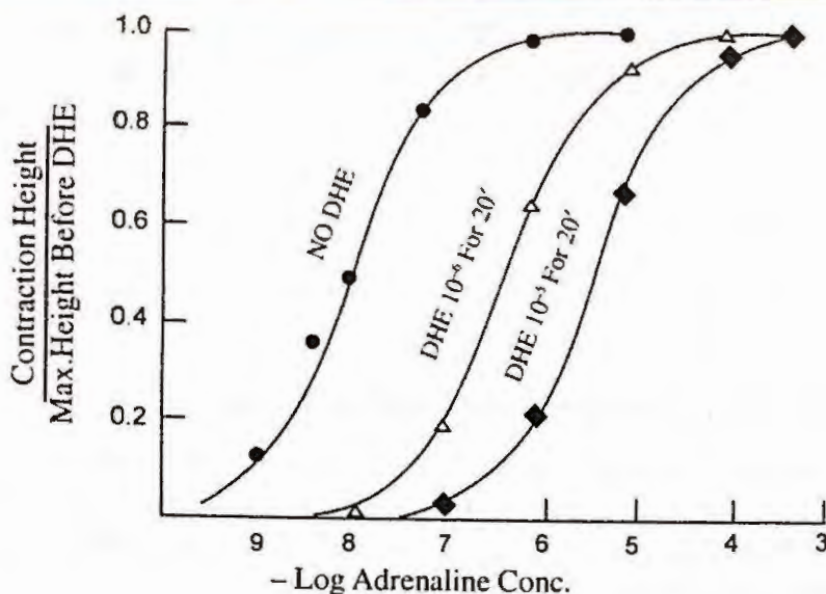


Fig. 19.4. Response of spiral strips of rabbit aorta to adrenaline in the absence and presence of dihydroergotamine (DHE). Parallel shift of the dose-response curve characteristic of competitive inhibition ( Furchgot, 1955).

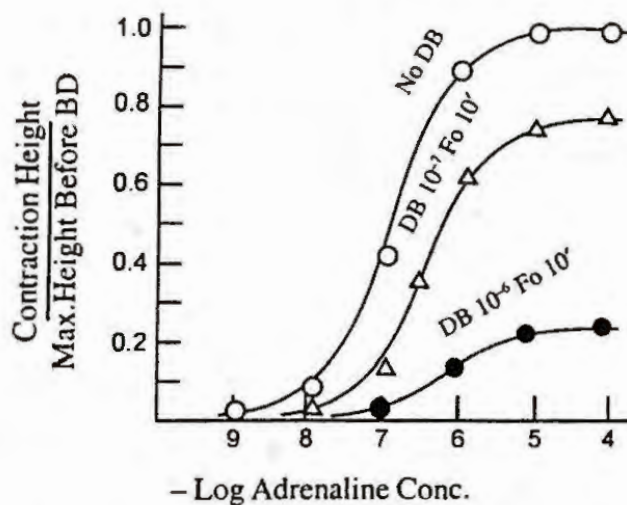


Fig.19.5. Response of spiral strips of rabbit aorta to adrenaline in the absence and in the presence of dibenamine (DB). Reduction in the slope as well as the maxima in addition to the shift of the dose-response curves suggestive of noncompetitive inhibition ( Furchgot, 1955).

### Noncompetitive Antagonism

An antagonist is said to be *noncompetitive* if it acts at a site different from that on the receptor with which the agonist reacts. The effect of noncompetitive antagonist is similar to that of nonequilibrium competitive antagonist, where the antagonist cannot be displaced even with higher concentration of agonist.

### EXPERIMENTAL METHODS FOR DETERMINATION OF TYPES OF DRUG ANTAGONISM

Responses of drugs before and after antagonism can be compared either by measuring responses to same dose of agonist, or by measuring doses of agonist producing the same response. The latter type of comparison is, however, more meaningful.

The experimental methods of determining the competitive nature of antagonism are as follows:

#### 1. Parallel Shift of the Concentration - Action Curve

When the concentration-action curve of agonist shifts to the right without any depression of maximal response following an antagonist, it is likely that the antagonist is of a competitive nature. However, it needs further confirmation for establishing the competitive nature of antagonism.

#### 2. Double Reciprocal Plot of Lineweaver and Burk

Chen and Russell (1950) suggested that the type of antagonism should be determined by the method of Lineweaver and Burk, in which the reciprocal of the effect is plotted against the reciprocal of the dose. If the points lie on straight lines, and if the straight lines determined in the presence and absence of the antagonist, intersect on the line corresponding to infinite dose, the antagonism is said to be competitive. The maximum response is given by the intercept of the lines with the ordinate axis. For illustration see Fig.19.6

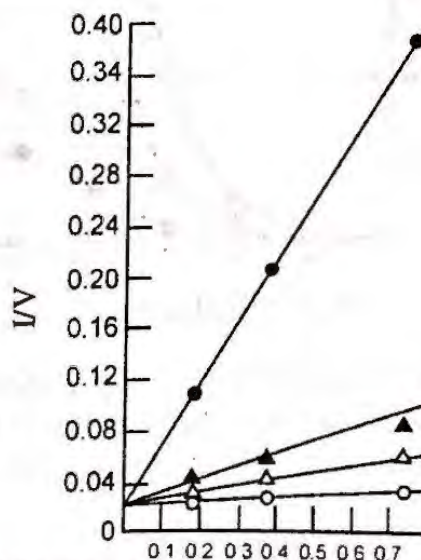


Fig. 19.6. Reciprocal analysis of the pressor effect of noradrenaline in the cat. Ordinate:  $1/V$ , where  $V$  is the pressor effect (mmHg). Abscissa:  $1/S$ , where  $S$  is the dose of noradrenaline ( $\mu\text{g}/\text{kg}$ ); o—o control;  $\Delta$ — $\Delta$  after chlorpromazine (7.2 mg/kg);  $\blacktriangle$ — $\blacktriangle$  after chlorpromazine (14.4 mg/kg);  $\bullet$ — $\bullet$  after chlorpromazine (28.8 mg/kg). Regression lines for the four sets of conditions have different slopes but a common intercept which lies on the line corresponding to infinite dose. This indicates that the inhibition caused by each of these doses of chlorpromazine is competitive (Gokhale *et al.* 1964).

3. Schild Plot

The most commonly used method for estimating  $pA_2$  values (*see later*) for pharmacological competitive antagonists is to plot  $\log (DR-1)$  against negative  $\log (I)$ , where DR represents the agonist dose-ratio, and (I) represents the molar concentration of the antagonist. When the Schild plot gives a statistically acceptable straight line with a slope, which is not statistically different from unity, then the antagonism is competitive (Fig. 19.7).

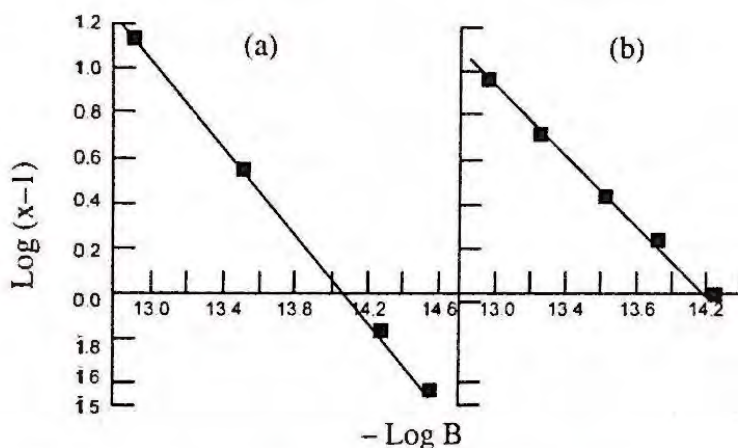


Fig.19.7. Results with the rat seminal vesicle preparation plotted by the method of Arunlakshana and Schild (1959) to determine the values of  $pA_2$  and  $pA_2 - pA_{10}$ . Ordinate:  $\log (x-1)$ , where x is the ratio of equiactive doses of adrenaline (a) or noradrenaline (b) in the presence and in the absence of chlorpromazine. Abscissa: negative  $\log B$ , where B is the molar concentration of chlorpromazine. The lines are calculated regression lines, and are very highly significant ( $P < 0.001$ ). They intersect the abscissa at 14.08 (a) and 14.2 (b) which are the  $pA_2$  values respectively for antagonism of adrenaline and noradrenaline responses. The  $pA_2 - pA_{10}$  values are 0.99 in (a) and 1.19 in (b); these values are in good agreement with the theoretical value of 0.95 for competitive antagonism. (Gokhale *et al.*, 1964).

4. Difference between  $pA_2$  and  $pA_{10}$  Values.

A method of testing competitive or noncompetitive nature of an antagonist is to determine both  $pA_2$  and  $pA_{10}$  values for the agonist-antagonist pair on the same tissue. If the difference between these two values is found to be 0.95 or very near (0.8 to 1.2), the antagonism is likely to be competitive. The two  $pA$  values may also be interpolated from the Schild plot line, and the difference calculated (Fig. 19.7). A gross departure of this difference from the value of 0.95 provides clear evidence that the inhibition is not competitive.

$pA_x$  VALUE

Schild (1947) proposed a  $pA$  scale (more or less analogous to pH scale) to express drug antagonism, so that comparison can reliably be made between drugs. He defined  $pA_x$  as the negative logarithm (to the base 10) of the molar concentration of an antagonist, which will reduce the effect of a multiple dose  $\times$  (usually 2 or 10)

of an active drug to that of a single dose. Thus, if the presence of a molar concentration of  $10^{-9.4}$  mepyramine in the bath reduces the effect of 2 mg histamine on guinea pig ileum to that produced by 1  $\mu$ g in the absence of mepyramine, the  $pA_2$  of mepyramine-histamine on guinea pig ileum is 9.4.

*Method of determination of  $pA_2$ .* The organ bath is connected with two bottles, one containing only the physiologic salt solution and the other containing the antagonist solution. A number of submaximal contractions are first obtained with an agonist at regular intervals till a constant response is produced. The organ bath is then filled with the solution containing the antagonist and the dose of agonist doubled in its presence. The response sometimes increases initially but it gradually diminishes and usually reaches a steady level in about 14 minutes. A concentration of antagonist that produces response slightly greater than the original response to a single dose of the agonist is first used, and then the bath is connected to the bottle containing the salt solution to allow for the complete recovery of its effect. The assay is concluded by producing a series of maximal contractions. Then the experiment is repeated with a concentration of antagonist that produces slightly smaller response than the original response to a single dose of the agonist. A fresh tissue for each concentration of antagonist is usually used particularly when the effect of antagonist is persistent. The contact time with antagonist may also be set at two or five minutes instead of 14 minutes. The result of a typical experiment is shown in Fig. 19.8.

*Plotting of  $pA_2$  values.* The contraction height of the single dose of agonist in the

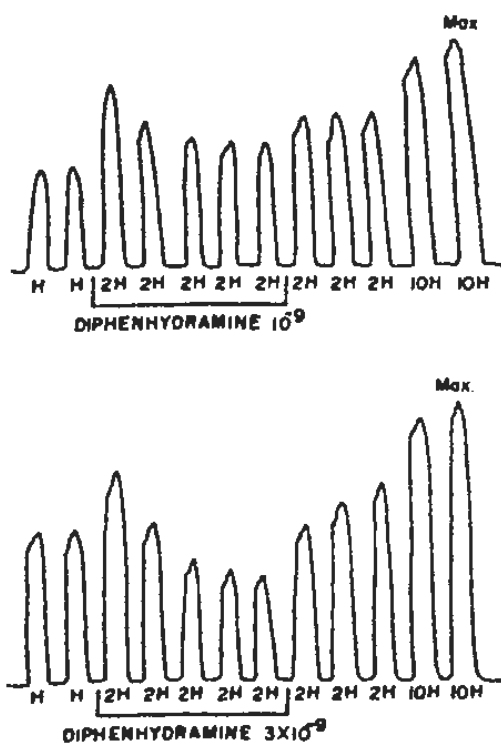


Fig. 19.8.  $pA_2$  determination on isolated guinea pig ileum. For details see text.

absence of the antagonist and of double dose of agonist following two concentrations of antagonist are expressed as percentage of maximal contraction; the latter two percentage contractions are then plotted against the two doses of antagonist on a logarithmic scale. The concentration of antagonist that produces response equal to single dose of agonist is found out by interpolation.

Alternatively, the responses in the presence of two concentrations of antagonist are expressed as percentage of the effect of a single dose in the absence of the antagonist. The  $pA_2$  value is interpolated at 100 per cent level.

The  $pA_2$  values for each agonist-antagonist pair are determined in at least three to four experiments on tissues from different individual animals, and the mean value calculated along with the standard error.

Another method of determination of  $pA_2$  value is from the Schild plot (Table 19.2). The steps are as follows:

1. Plot log dose-response curves in the absence and in the presence of antagonist.
2. Determine dose-ratio (DR) from these curves for each concentration of antagonist (Fig 19.3).
3. Plot  $\log (DR - 1)$  as ordinate against either log molar concentration or against negative log molar concentration of antagonist.

**Table 19.2**  
**Computation of values for Schild plot from results of**  
**atropine-acetylcholine antagonism on mouse isolated ileum**

<i>Atropine (molar conc.)</i>	<i>log molar</i>	<i>DR</i>	<i>(DR - 1)</i>	<i>log (DR - 1)</i>
$1.44 \times 10^{-9}$	9.16	2.75	1.75	0.24
$4.32 \times 10^{-9}$	9.64	17.38	16.38	1.21
$1.30 \times 10^{-8}$	9.11	138.00	137.00	2.14

4. In the latter case, the  $pA_2$  value is directly read out at the point where the line intersects the abscissa at the zero level of the ordinate, while in case of the former, the log value thus obtained is converted to negative logarithm in order to arrive at the  $pA_2$  value (Fig. 19.9).

The  $pA_2$  values of four antagonists against histamine and acetylcholine as measured on the guinea pig ileum are presented in Fig. 19.10.

A high specificity of antagonists is indicated by high values, while low specificity gives low values. The points on the two scales referring to the same antagonist are joined. If an antagonist does not discriminate between histamine and acetylcholine (i.e. nonspecific antagonism) the line joining the two scales tends to be horizontal (e.g. pethidine); if it discriminates sharply, the line is steep (e.g. mepyramine, atropine).



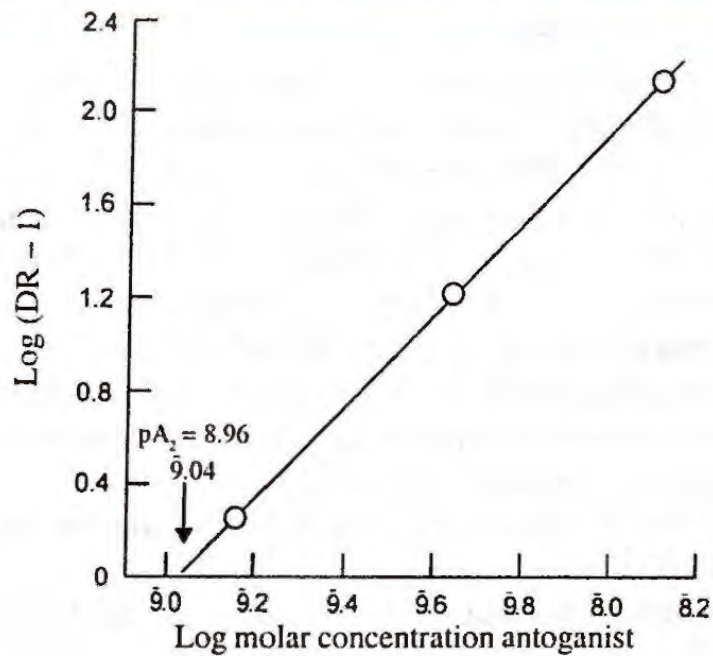


Fig. 19.9 Determination of  $pA_2$  value from the Schild plot. For details text.

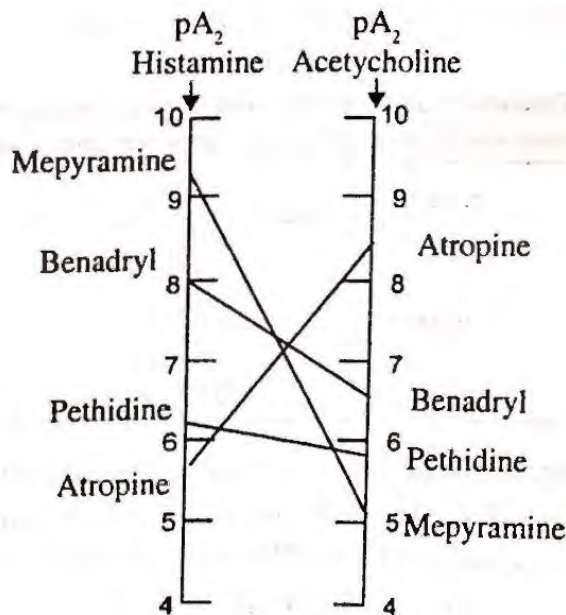


Fig. 19.10.  $pA_2$  scale showing the activities of four antagonists against histamine and acetylcholine as measured on the guinea pig ileum (Schild, 1947). For details see text.

The quantitative applications of  $pA$  measurements are as follows :

1. Drugs that act on the same receptors can be expected to be antagonized by the same concentration of a competitive antagonist, even though they themselves may differ considerably in activity. For example, histamine and pyridylethylamine differ markedly in their stimulant activity on guinea

## Quantitative Study of Antagonists on Isolated Preparations

pig ileum (the former being about 100 times more potent than the latter); nevertheless, they are antagonized by exactly the same concentration of diphenhydramine giving the same  $pA_x$  values.

2. If a given drug-antagonist pair gives similar  $pA_x$  values in different preparations, it is suggestive though not a positive proof that the receptors are identical. On the other hand, different  $pA_x$  values are indicative of different receptors (Table 19.3).
3.  $pA_x$  values can be used to distinguish between competitive and noncompetitive antagonists.

### $PK_B$ VALUE

Sometimes, the term  $pK_B$  value is used to refer to the negative logarithm of the estimated dissociation constant ( $K_B$ ) having the same connotation as the  $pA_2$  value.

Table 19.3  
 $pA_x$  values in different preparations†

Preparation	Atropine - Acetylcholine		Mepyramine - Histamine	Diphenhydramine - Histamine
	$pA_2$	$pA_{10}$	$pA_2$	$pA_2$
Guinea pig ileum	8.8	8.1	9.3	8.0
trachea	-	-	9.1	7.8
lung (perfused)	8.8	7.6	9.4	7.8
Rat intestine	-	8.1	-	-
Frog rectus	-	4.2*	-	-
auricle	-	8.3	-	-
Chick amnion	8.8	-	-	-
Human bronchi	-	-	9.3	-

† Schild, 1961.

\* Strikingly different value is presumably due to different (nicotinic) receptors in frog rectus.

### $PD'_2$ VALUE

While  $pA_2$  is a measure of the affinity of a reversible competitive antagonist for a specific receptor,  $pD'_2$  is a measure of the affinity of a reversible noncompetitive as well as an irreversible competitive antagonist for a specific receptor.  $PD'_2$  is defined as the negative logarithm of the molar concentration of noncompetitive antagonist which will reduce the effect of an agonist to one-half (50%) its maximum (Ariens and van Rossum, 1957).

$pD'_2$  value is determined as per the following equation:

$$pD'_2 = pD'_x + \log [(E_{Am}/E_{\Delta 8m}) - 1]$$

where  $pD'_x$  is the negative logarithm of the molar concentration of the antagonist

employed, and  $E_{Am}$  and  $E_{ABm}$  are the maximal contraction heights in the absence and in the presence of the antagonist respectively (Bickerton, 1963).

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# Anaesthetized Animal Preparations

## RECORDING OF THE BLOOD PRESSURE

Recording of the blood pressure in an anaesthetized animal is one of the standard methods for the pharmacological studies of drugs.

A dog, cat or rabbit is anaesthetized with a suitable anaesthetic. A midline incision is made on the skin of the neck starting from the lower end of larynx up to the upper end of thorax. Muscles are separated along the midline with the help of a pair of scissors by introducing the closed tips and then separating the blades. Trachea is exposed by retracting the pretracheal muscles, and a transverse cut is made in between the two rings. A tracheal cannula is introduced into the gap between the two rings pointing towards the lungs and held firmly in position with the help of a ligature. The purpose of cannulating the trachea as a routine is to allow free breathing without any obstruction by secretions that can be cleared as and when necessary, and to provide artificial respiration when needed. The volume of air per stroke of the respiratory pump and the rate are adjusted depending on the species of the animal. Carotid arteries that lie close to the trachea on either side along with the veins and the nerves are easily recognized by their elastic and pulsating nature. One of these arteries is cleaned from the accompanying structures for a sufficient length with the help of a blunt dissector. It is then tied as near the head end as possible, a bulldog clamp is placed about 3 cm nearer the head and a thread is passed round the artery. A cut is made carefully on the artery close to the ligature with the help of a sharp curved scissors so as to make a small opening through which an arterial cannula already filled with some anticoagulant fluid is inserted directing towards the heart, and firmly secured with the help of the ligature already in position. The three-way tap in the manometer is then turned so as to fill one limb, the connecting tubing and the cannula with the anticoagulant fluid from the reservoir bottle. The pinchcock on the short tubing attached to the side tube of the artery cannula is opened at the same time so as to allow the coagulant fluid to run out. When the whole system is rendered free of air bubbles the pinchcock is closed. The pressure in the manometer is then increased to about 150 mmHg and the three-way tap turned so that the manometer now remains in communication only with the cannula. The positive pressure being approximately equal to that of the blood in the animal, it prevents too much blood from coming out of the animal's artery into the recording system. Before the bulldog clamp is removed, 0.5 ml of

1% heparin solution is injected into the arterial cannula through the rubber tubing. The bulldog clamp is then taken off; the column of mercury rises or falls slightly until its pressure counterbalances that of the blood. The writing point remains at a constant level except for slight oscillations due to the heart beats and the respiratory movements. The height of the mercury column midway between the top and bottom of these oscillations is taken as the mean arterial pressure. The femoral vein is exposed by a midline incision on the medial surface of the upper part of the thigh. In the rabbit, usually the external jugular vein is exposed just under the skin at the side of the neck taking special care not to damage it. The venous cannula is inserted into the vein in the same fashion as into the artery except that the bulldog clamp is first applied proximally and a ligature tied a little distally while the vein is full with blood. After the cannula is tied in position, it is connected with a burette filled with warm saline. Drugs are injected through the rubber tubing close to the cannula and a constant volume of saline is allowed to run each time after injection.

*Normal variations in the blood pressure record.* A steadily rising base line when the blood in the arterial cannula appears dark suggests anoxia. Occasionally, after switching to artificial respiration a spiky base line may occur. This is often an indication that the animal is trying to breathe against the artificial respiration. An erratic base line also occurs when the anaesthesia is too light or the bladder is full. The tracings of the normal blood pressure record show that in addition to the variations in pulse pressure (waves of the first order due to heart beats) there are sometimes variations with respiration (waves of the second order). The blood pressure somewhat rises with each inspiration and drops with each expiration due to an increased blood flow to the heart during inspiration, concomitant acceleration of heart and spread of the stimulation from the respiratory to the vasomotor centres producing slight vasoconstriction. Very large respiratory waves (Traube-Hering curves) are sometimes observed during abnormally slow respiration caused by 'explosions' of the strong excitation of the respiratory centre. The blood pressure curve sometime shows slow waves each taking in a period of eight or more respiratory movements (waves of the third order or Meyer's waves). They are dependent on changes in nervous control of blood vessels; rise when insufficient blood flows to the cerebrum, or during increase in the intracranial pressure. These are also commonly seen after haemorrhage due to the following mechanism. As the blood pressure falls due to haemorrhage, chemoreceptor stimulation occurs due to stagnant anoxia of the chemoreceptor glomus tissue resulting in reflex vasoconstriction. The systemic pressure thereupon rises and as a result the chemoreceptor stimulation dies away as the blood flow to the glomus cells improves. This results in a reduction in the reflex vasoconstriction producing a fall in blood pressure once again thus repeating the cycle. These Meyer's waves are always accentuated by bilateral carotid occlusions and are abolished by cutting the chemoreceptor nerves.

### RECORDING OF NICTITATING MEMBRANE CONTRACTIONS

Cat is more suitable than the dog for recording of nictitating membrane contractions, because the former has a highly developed nictitating membrane that responds to the stimulation of the cervical sympathetic trunk. In case of the dog, it is less developed; in addition the cervical sympathetic and vagal nerves run inseparably in the same trunk (vagosympathetic nerve); thus stimulation of the cervical sympathetic cannot be obtained independent of central vagal stimulation. Nictitating membrane contains smooth muscle with a low resting tone. Hence, it is not capable of exhibiting relaxation.

Chloralose is a suitable anaesthetic because of its smaller inhibitory action on autonomic functions, greater constancy of the depth of anaesthesia produced by it, and the resulting steadiness of the nictitating membrane tone and the blood pressure.

After cannulation of the trachea, the left carotid artery and the right femoral vein, the head of the cat is raised on a wooden block about 10 cm high and the ears pinned on to the block. The head is then held rigidly by passing a rod (about 4 mm dia) between its jaws and tying them firmly together with a string. The ends of the rod are gripped tightly in clamps supported on uprights fixed on the sides of the table. By means of a fine needle a silk thread is passed through the centre of the edge of the right nictitating membrane (i.e. opposite side of the cannulated carotid artery) and tied; the free end of the thread is then taken outward and forward making an angle of about  $30^\circ$  with the axis of the cat, then round a pulley and vertically to a frontal writing lever (magnification 6 to 7 times, load 2g). The superior cervical ganglion and the preganglionic and postganglionic cervical sympathetic nerves on the right side are traced along the course of the carotid artery. The cervical sympathetic chain is dissected. A silk thread tied tightly around it and the chain is cut low in the neck so that a length can be laid on a pair of shielded electrodes and left in position.

Square wave shocks from an electronic stimulator are used to stimulate the nerves. When supramaximally effective voltage and pulse width of 0.5 to 1 msec are used (*see* Chapter 5), the responses usually remain fairly constant in experiments lasting for almost eight hours or longer. The magnitude of the contraction varies with the frequency of stimulation between 0.1 to 10 shocks per second. If the frequency is increased further (up to 30/sec) the magnitude of contraction usually increases, but because of ganglion fatigue the contractions are poorly maintained and subside to a level not exceeding that produced by 10 shocks per second. The choice of the frequency of stimuli and the duration of their application depends on the nature of the investigation. If the lever ratio is 15:1 and tension 7 g, a maximum contraction should record a height of about 10 cm.

### PREPARATION OF SPINAL CAT BY DALE'S METHOD

An adult cat is anaesthetized with ether, the trachea cannulated, and the

anaesthesia maintained through the cannula. Both carotids are clamped temporarily with bulldog clamps. The animal is then turned over the belly; skin is incised down the midline from the top of the head up to the level of shoulders by holding the head with the left hand and flexing the neck, and the two flaps of skin are held back with retractors. The first layer of muscles is then divided down the midline from the skull for about 7 cm and held apart; muscles are cut away from the sides of the spine of second cervical vertebra (having most prominent spine) avoiding the skull end of the spine. Muscles are cleared from the bone by using a thin blunt dissector, and held away by hooks and weights. Muscle attached to the lower end of the spine is cut through, the spine cut off with a pair of bone forceps. To minimize the loss of blood at this stage the animal should be as deeply anaesthetized as possible. Bleeding from the bone may be stopped with plasticine and from other tissues by pressing cotton wool swab dipped in hot saline and wrung out. With a pair of bone forceps the layer of bone covering the spinal cord is carefully nibbled away till the dura mater is exposed over a length of about 1 cm and over its full breadth. The dura and spinal cord are then cut through with a sharp instrument; anaesthetic removed, and artificial respiration begun immediately. The brain is destroyed with a probe (stiff wire 3 to 4 mm dia) thrust through the foramen magnum, withdrawn and a tapering cone of plasticine introduced into the brain, and finally the foramen is plugged with a small cork. The upper end of the cut cord should be separated by about 1 cm from the lower end of the cork. Any bleeding from the end of the cord is arrested by applying cotton wool dipped in hot saline. Skin of the back of the neck is finally sewn together, and the cat turned on its back.

The temperature of the cat is maintained by heating the operating table with the help of electric lamps. The blood pressure, which may be as high as 150 mmHg initially, usually falls to a lower level during the course of one hour and maintained for 24 hour. The preparation can be used after one hour; if the blood pressure is high or variable the spinal cord should be destroyed by passing a stiff wire down the spinal canal and plugging the open end of the canal with plasticine to minimize bleeding. The destruction of the spinal cord causes a precipitous fall in blood pressure; in order to keep the cat alive, an intravenous injection of adrenaline should be made promptly.

### RECORDING OF BLOOD PRESSURE IN ANAESTHETIZED RAT

An anaesthetized rat is placed supine on a small-animal operating board and secured by tying the limbs. A midline incision is made on the neck; the trachea exposed and cannulated to ensure a free airway. The common carotid artery on one side is then exposed and ligated at the superior end and clamped at the inferior end. A polythene cannula attached to a 23-gauge needle is inserted into the artery through a small incision and connected via a three-way stopcock to a Condon mercury manometer and to the pressure bottle of the mercury manometer system filled with 0.9% NaCl solution containing heparin 1000 units per ml. The system is

## Anaesthetized Animal Preparations

flushed briefly with heparin saline solution and blood pressure recorded by releasing the arterial clamp. The external jugular vein is exposed on the other side and cannulated with a polythene tubing attached to a 23-gauge needle and connected via a three-way stopcock for injecting drugs and for flushing with saline-heparin solution after each injection.

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# Assay of Drugs on Blood Pressure of Anaesthetized Animals

## EFFECT OF THE AUTONOMIC DRUGS ON BLOOD PRESSURE

A common observation in blood pressure experiment is that a dose of a drug sometimes produces a small rise and sometimes a big rise in blood pressure depending on whether the initial blood pressure is high or low respectively. This is according to the *law of initial value of Wilder*, which states that given a standard stimulus or drug and a standard period of measurement, the higher the initial value, the smaller is the response to function-raising stimuli, and greater is the response to function-depressing stimuli; the converse occurs when the initial value is low. Further beyond a certain range of initial value there is an increasing tendency to exhibit no response (occasional) or a reversal of response (frequent). The higher the initial values, the more frequent the reversals with function-raising stimuli; the lower the initial value, the more frequent the reversals with function-depressing stimuli. For instance, a vasomotor reversal with adrenaline is demonstrable in a chloralosed cat but not in a spinal cat, because in the latter the blood vessels are already maximally or almost maximally dilated so that adrenaline cannot dilate them further.

*Adrenaline on blood pressure.* When adrenaline is injected intravenously into an anaesthetized animal, the blood pressure rises rapidly to a peak and then falls below the initial level (base line) before returning to the base line (i.e. biphasic response, cf: noradrenaline). The mechanism of the rise in blood pressure is due to (a) a direct myocardial stimulation (positive inotropic), (b) an increased heart rate (positive chronotropic), and (c) arteriolar constriction in many vascular beds especially in skin, mucous membrane and splanchnic region. The pulse rate at first accelerated becomes markedly slow at the height of the rise in blood pressure due to the compensatory reflex through vagus. This bradycardia is absent after vagotomy or after atropine. Minute doses of adrenaline (less than 0.1  $\mu\text{g}/\text{kg}$ ) may cause the blood pressure to fall below normal level without any initial rise. The depressor effect of small doses and the biphasic response to larger doses are due to the greater sensitivity to adrenaline of  $\beta$ -receptors in vascular beds (i.e. of skeletal muscles which are dilated) than of  $\alpha$ -receptors in vascular beds (i.e. of skin, mucosa, splanchnic and kidney which are constricted). Doses of adrenaline too small to act

on  $\alpha$ -receptors, lower the blood pressure by their  $\beta$ -receptor action; larger doses activate  $\alpha$ -receptors predominantly and the overall effect of full activation of both  $\alpha$  and  $\beta$ -receptors is an increase in peripheral resistance with a consequent rise in blood pressure. When enough adrenaline has been eliminated to end its effect on the less sensitive but predominant  $\alpha$ -receptors, the effect on  $\beta$ -receptors persists for a time, thus accounting for the secondary fall of the biphasic response.

As early as in 1906, Henry Dale demonstrated that in anaesthetized cat when ergot preparation is administered prior to a dose of adrenaline, instead of a rise in the blood pressure there is a fall. Subsequently it was established that the fall in the blood pressure was due to the blocking of  $\alpha$ -receptors by ergot or related compounds, so that when adrenaline is administered following these agents it fails to excite  $\alpha$ -receptors, but stimulates the  $\beta$ -receptors leading to the fall in the blood pressure. This phenomenon is known as 'vasomotor reversal of Dale'.

*Acetylcholine on blood pressure.* Acetylcholine in a small dose (2  $\mu$ g) produces a fall in blood pressure due to vasodilatation without any change in heart rate, while a large dose (50  $\mu$ g) produces an abrupt fall accompanied by bradycardia due to cardio-inhibition in addition to vasodilatation. In the presence of eserine (0.1 mg at 1 min intervals for 3 doses) a small dose (2  $\mu$ g) of acetylcholine now causes prolonged fall in blood pressure as well as slowing of the heart rate. Following 2 mg atropine even larger dose of acetylcholine (50  $\mu$ g) fails to produce any response. Still higher doses of acetylcholine (2.5 to 5 mg), however, produce a steep rise in blood pressure due to its nicotinic action on the sympathetic ganglia producing vasoconstriction, followed by a second rise accompanied by an acceleration of heart rate due to adrenaline released from adrenal medulla.

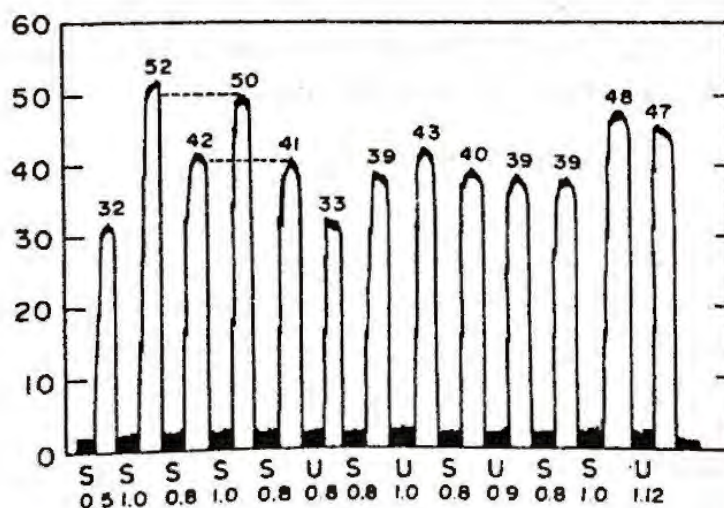
### ASSAY OF ADRENALINE

The blood pressure of the spinal cat provides the most sensitive preparation for the assay of adrenaline and noradrenaline. The chloralosed cat treated with hexamethonium (50 mg/kg) is a less sensitive preparation. The blood pressure in this preparation may be stabilized by the intramuscular injection of ergotamine tartrate (0.05 to 0.1 mg/kg), while cocaine hydrochloride (4 to 8 mg/kg i.m.) increases the sensitivity of the preparation.

The blood pressure of pithed rat is an extremely sensitive test object for the determination of very small amounts of adrenaline and noradrenaline (10 ng/ml). The hexamethonium treated rat (1 mg/100 g) anaesthetized with urethane can also be used, although the blood pressure is not as stable as it is in the pithed rat.

An anaesthetized dog can also be used. A medium-sized healthy dog is anaesthetized with a barbiturate and prepared for the recording of blood pressure. In order to increase the accuracy of the assay, the vagal cardio-inhibitory mechanism that brings about reflex bradycardia with each rise in blood pressure following adrenaline is blocked by atropine sulphate 1 mg/kg intravenously. Vagal paralysis is tested by injection of acetylcholine chloride 10  $\mu$ g/kg or by electrical stimulation

of vagus. If either of these still produces any fall in blood pressure, atropinization is incomplete, and subsequent doses of half the original dose of atropine should be given until the dose of acetylcholine or vagal stimulation elicits no fall. The standard and the test preparations of adrenaline are diluted in acid saline in sufficient concentration so that a rise in blood pressure equivalent to 30 to 60 mmHg is obtained following 0.5 to 1.5 ml of each preparation. The animal is then tested for sensitivity and for uniformity of responses by alternate injections of two dilutions of the standard solution that differ in amount by not more than 20 per cent and which produce elevations in blood pressure between 30 and 60 mmHg. A constant interval of not less than five minutes but long enough to allow the blood pressure to return to approximately its former level should be used. After two or more injections of each dose have been made alternately, the rise in blood pressure is measured to the nearest millimeter and the average rise and the maximum difference in the rise produced by each dose is calculated. If the two average rise differ by at least 5 mm, and this difference is at least twice the maximum difference in the rise produced by each dose, the animal is considered satisfactorily sensitive, otherwise this is discarded. A matching assay is then carried out (Fig. 21.1) as already described for isolated preparations (*see* Chapter 18). The dose of the standard is kept constant unless the sensitivity of the animal changes, in which case the dose is increased or decreased so as to give a rise of blood pressure of between 30 and 50 mmHg.



21.1. Matching assay of unknown solution of adrenaline on the blood pressure of anaesthetized dog. Ordinate: mmHg rise from basal level. S – standard preparation of adrenaline (ml). U – test preparation of adrenaline (ml). For details see text.

#### ASSAY OF ACETYLCHOLINE

An anaesthetized cat or dog is prepared for the recording of the blood pressure. Before starting the assay, mepyramine 1 mg/kg is injected intravenously. If breathing

is regular and blood pressure record is free from disturbing fluctuations, the animal is ready for the assay; if not, artificial respiration is given. If acetylcholine 2 ng/kg i.v. does not produce a perceptible fall in blood pressure, eserine 0.05 to 0.1 mg/kg i.v. is injected and 10 minutes allowed for full sensitization. Sometimes, there is not much improvement of the acetylcholine effect even after eserine. Excessive eserization should be avoided because of the danger of progressive circulatory failure. A matching assay is carried out allowing a dose interval of two to three minutes. At the end of the assay, it should be demonstrated that the depressor effect of the extract is destroyed by alkalization and that it is antagonized by atropine. The threshold dose of acetylcholine is 1 to 6 ng for a 2 kg cat, and the accuracy of the assay is usually about 10 per cent. It is the most rapid method when many samples are to be assayed and is about equal to leech sensitivity.

### ASSAY OF HISTAMINE

A cat or dog of suitable size is anaesthetized with chloralose or barbiturate and prepared for recording of the blood pressure. Sensitivity of the animal to histamine is determined by injecting doses of standard solution at five-minute intervals. Doses corresponding to 0.05, 0.1 and 0.15  $\mu\text{g}$  of histamine base per kg body weight are injected intravenously. A fixed dose of the standard producing a fall in pressure of about 20 mmHg is injected with changing doses of the unknown at regular intervals, and a matching assay is carried out. This may be accomplished by exact matching of the unknown and the standard provided larger and smaller falls have also been obtained.

The depressor responses of histamine can be inhibited by antihistamine as illustrated in Fig. 21.2.

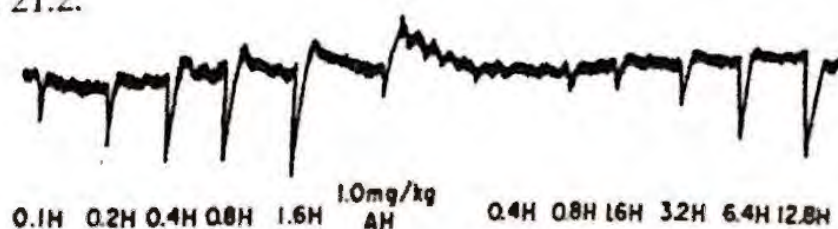


Fig. 21.2. Antagonism of the depressor response of histamine (H) by antihistamine (AH). Doses of H in  $\mu\text{g}$ . For details see text.

In this experiment, a dose of 3.2  $\mu\text{g}$  histamine after 1 mg/kg of an antihistamine produced about the same effect as 0.2  $\mu\text{g}$  before blocking, and a dose of 6.4  $\mu\text{g}$  after blocking produced about the same response as 0.4  $\mu\text{g}$  before blocking. Hence, the dose-ratio was about 16.

### DETERMINATION OF ED50 VALUE

As with *in vitro* experiments, the geometric rather than arithmetic means of equieffective (ED50) doses should be determined. Unlike *in vitro* experiments, it is

not always possible to reach a maximum response *in vivo* experiments. In such cases, doses producing a specific magnitude of responses ED<sub>40</sub> mmHg, are calculated; that is, a dose that causes a pressure rise of 40 mmHg.

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# Identification of A Compound by Testing on Anaesthetized Animals

## TESTING ON BLOOD PRESSURE

For investigating a new compound, a series of *standard tests* are carried out on blood pressure of the anaesthetized animal before as well as after injecting the compound. These tests which constitute the *normal response brackets* are: (a) electrical stimulation of the peripheral vagus nerve (20 shocks/sec for 2 to 5 sec), (b) injecting acetylcholine (0.5 to 5.0  $\mu\text{g}/\text{kg}$ ), (c) injecting noradrenaline (1 to 5  $\mu\text{g}/\text{kg}$ ), (d) injecting adrenaline (1 to 5  $\mu\text{g}/\text{kg}$ ), and (e) occluding both carotid arteries for 30 or 45 seconds. Vagal stimulation and acetylcholine injection produce a transient fall in blood pressure, while injections of adrenaline and noradrenaline a transient rise.

*Carotid occlusion reflex.* Two factors are responsible for the rise in blood pressure following occlusion of one or both carotid arteries: (a) reduction in baroreceptor impulses owing to the reduced pressure in the region above the point of occlusion, and (b) increased chemoreceptor activity resulting from the hypoxic conditions within the carotid body due to reduced blood flow. For centrally mediated cardiovascular reflexes, it is desirable to keep anaesthesia light or to use anaesthetics having minimal depressant action on the autonomic responses. Chloralose in this respect is satisfactory in the cat, and morphine and chloralose in the dog. Centrally mediated *vasopressor reflexes* (carotid occlusion reflex) are more susceptible to the blocking action of most drugs than are centrally mediated *vasodepressor reflexes* (stimulation of cut central end of vagus in most animals, or of depressor nerve in rabbits). The afferent nerves that arise in the carotid sinus pass along a branch of the glossopharyngeal nerve to the cardiac centres in the medulla oblongata. The cardio-aortic nerve endings arise from the left ventricle and the aortic arch ascending along the trunk of the vagus in man and in most animals but forming a separate nerve bundle (depressor nerve) in the rabbit. In the cat the depressor nerve may run with the vagus trunk on the right but separately on the left. Stimulation of the central end of the cut vagus produces cardiac slowing and a fall in blood pressure as a result of afferent impulses traveling along the stimulated vagus nerve to the cardioinhibitory centre, and efferent impulses passing down the other intact vagus nerve to the heart. When both the vagi are cut, there is no



## Identification of A Compound by Testing on Anaesthetized Animals

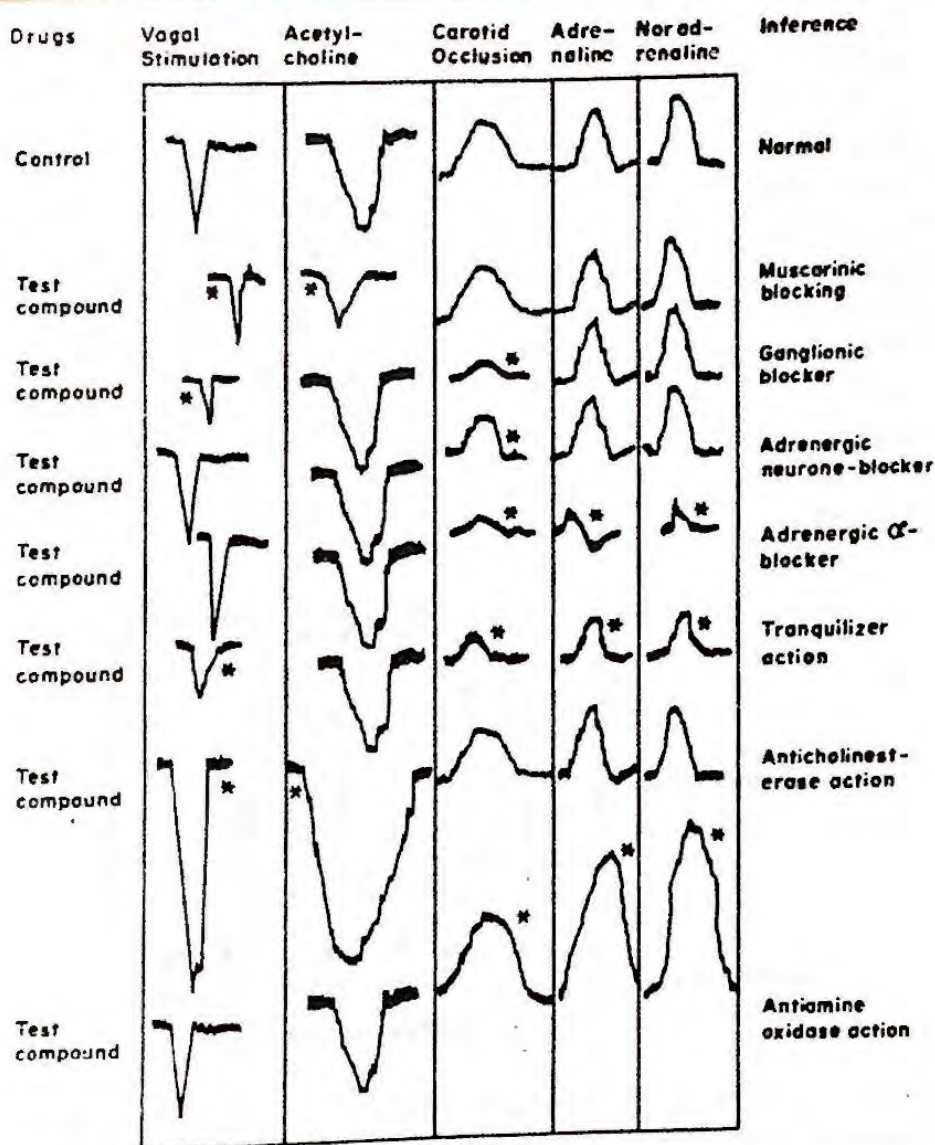


Fig. 22.2. Identification of a compound by observing changes in 'normal response brackets'. \* Indicates alteration in the responses.

Similar changes are also observed when the corresponding cervical sympathetic ganglion is removed by prior operation two to three weeks earlier.

*Effect of ganglionic blocking agents.* Following hexamethonium or related drugs, responses to preganglionic nerve stimulation (sympathetic chain) are blocked, while responses to postganglionic nerve stimulation remain unaffected. This is strikingly demonstrable by recording the contractions of both nictitating membranes, stimulating the preganglionic nerve on one side and the postganglionic nerve on the other before and after injecting hexamethonium 0.25 mg/kg. A characteristic of these compounds is that a dose that fails to block the nictitating membrane contractions caused by a brief burst of stimulation (5 sec) of the



preganglionic nerve, produces a fall in the contraction during continuous stimulation (60 sec), that is, the membrane fails to maintain a contraction (Fig. 22.3). The most sensitive situation is that in which the compounds are injected during continuous stimulation of the preganglionic nerve with 5-20 shocks/sec (supramaximal) of 0.5 msec duration, or during intermittent stimulation (e.g. for 30 sec every min). For compounds with a slow onset of action, or in preliminary testing of new compounds, it is better to study responses to a fixed frequency (10 shocks/sec) applied for, say, one minute at intervals of about 10 minutes or longer. Local anaesthetic, such as procaine, which is equiactive with hexamethonium against nicotine-induced contraction of isolated guinea pig ileum has only about one-fortieth of the activity of hexamethonium on the nictitating membrane, and has different effect on the blood pressure. Hence, it is easily distinguishable from the true ganglionic blocking agents.

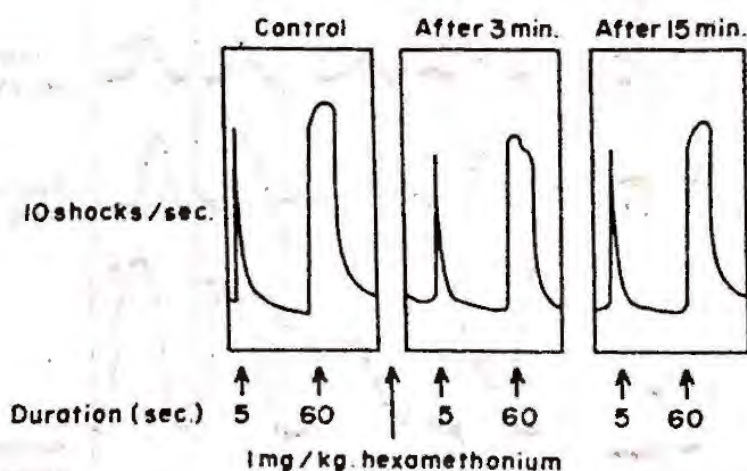


Fig. 22.3. Nictitating membrane contractions in chloralosed cat. Following hexamethonium, the reduction of response to brief stimulation was small, but it failed to sustain a contraction during continuous stimulation for 60 sec. (Green and Boura, 1964)

*Effect of adrenergic neuron blocking agents.* The preganglionic cervical sympathetic nerve is stimulated for one-minute period (0.3 to 30 shocks/sec) during which the contractions of nictitating membrane usually attain the peak. Contractions though reduced by adrenergic neuron blocking agents are well maintained during continuous nerve stimulation (cf. ganglion blockers which produce 'spike contractions'). They also increase the response of nictitating membrane to adrenaline and noradrenaline. It is also desirable to test the drug against responses to more than one frequency of stimulation, and to plot the regression curve relating the nictitating membrane contractions to log of the frequency of nerve stimulation (say, 0.3, 1, 3, 10 and 30 shocks/sec). Guanethidine produces a shift to the right of this regression curve while bretylium produces a reduction in the slope.

## Identification of A Compound by Testing on Anaesthetized Animals

*Effect of adrenergic  $\alpha$ -receptor blockers.* Contractions due to both pre and post-ganglionic sympathetic nerve stimulation are inhibited following  $\alpha$ -adrenoceptor blockers (cf. ganglion blockers). Responses to adrenaline and noradrenaline are also inhibited (cf. adrenergic neuron blockers) usually more than to nerve stimulation.

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# Identification and Estimation of Biologically Active Substances

The body fluid or tissue extract is subjected to physicochemical methods prior to the biological test primarily for two reasons: (a) concentrating the fluid (e.g. urine) so that the activity becomes measurable, and (b) eliminating unwanted active substances that may interfere with the assay. Most methods of extraction involve a certain variable percentage loss of activity. This however, can be determined by adopting the similar method of extraction with a measured amount of the active substance expected in the extract. The final fraction is tested biologically, and the percentage of recovery of the substance added determined. The greater the sensitivity of the assay and the higher the degree of concentration of the extract, the more likelihood that the interfering substances will show themselves. It is also possible that other substances in the extract may modify the properties of the active substance. This is checked by adding a known substance to the extract in quantities that might be present initially, and then testing the properties of the known substance under similar condition as those of the unknown substance. If their properties still differ they cannot be identical.

If a *specific tissue* can be found which is very sensitive to one of the substances in an extract and insensitive to others, this can be utilized for identification. For instance, acetylcholine can be assayed by its effect on the frog rectus muscle, which does not respond to histamine, 5-hydroxytryptamine, substance P or adenosine; or on rabbit intestine which is insensitive to histamine and inhibited by adenosine. Rat jejunum is suitable for screening of muscarinic substances, since in contrast to guinea pig ileum it responds feebly to nicotine and other ganglionic stimulants.

When a *specific antagonist* is available, the same may be utilized for the identification of the agonist. Since a high concentration of any antagonist will make the tissue insensitive to any agonist (nonspecific), it is desirable to include two control drugs for comparison, one of which is antagonized by a specific concentration of the antagonist while the other is not. Equiactive doses of two control drugs and the extract are given first and then repeated in presence of the antagonist. If the extract is antagonized along with one control, these two are given alternately in equiactive doses. If the two substances are identical, the effects of the two will be equally depressed by a moderate dose of the antagonist, and after washing they will recover together in a similar fashion (Fig.23.1). When specific

antagonist is not available for a substance, an alternative method is to add a suitably high concentration of the substance into the bath after which there will be a contraction followed by relaxation in spite of the continued presence of the substance. It is then insensitive to small doses of the same substance already present in high concentration but responds to various other stimulant substances. However, if a very high concentration of the first substance is used a non-specific desensitization may occur.

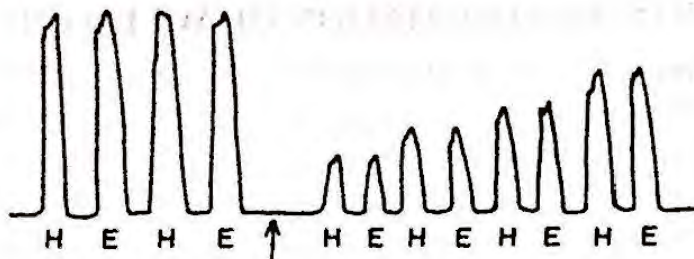


Fig. 23.1. Contractions of guinea pig ileum to equiactive doses of histamine (H) and tissue extract (E). Following antihistamine at the arrow, there is almost equal inhibition and recovery of both the responses.

To establish a definite identity of a substance, *parallel quantitative assay* using different pharmacological tests are carried out, and relative potencies are found out in comparison to the standard. If several such parallel estimates of the potency of the substance agree quantitatively with one another, then the identification of the active substance can be taken as established (Fig. 23.2). The ratio of the results of two parallel assays is called the *index of discrimination*. For substances to be

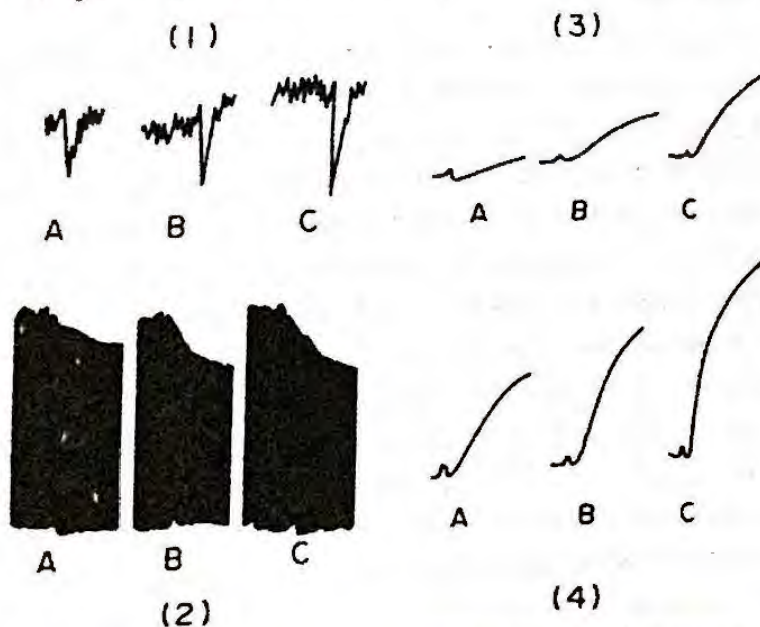


Fig. 23.2. Identification of released acetylcholine by parallel quantitative assays on (1) blood pressure of cat, (2) isolated frog heart, (3) isolated frog rectus, and (4) isolated dorsal muscle of the leech. In each test, the effluent (B) had the same activity relative to the standard acetylcholine solutions (A and C). (Dale and Feldberg, 1934)

identical with each other the index would be about 1; an index of 10 or more makes it extremely unlikely that the substances under test are the same.

Before a substance is rejected as inactive, one has to be certain about sensitivity of the test object, and that a sufficient amount of the substance has been used for the test. If the effects of an extract cannot be fitted with those of any known compound, a possibility of presence of more than one active substance should be thought of.

### DETECTION AND ESTIMATION OF ACETYLCHOLINE

A number of tests are available for identification of acetylcholine as follows:

1. Parallel quantitative assay giving almost identical results on a number of test objects, such as guinea pig ileum, rabbit intestine, and blood pressure in anaesthetized cat or rat, all producing muscarinic action antagonized by atropine; while rectus abdominis muscle of frog, and dorsal leech muscle producing nicotinic action antagonized by curare. All these effects are potentiated by eserine. When guinea pig ileum is used for testing the presence of acetylcholine, the bathing fluid should contain mepyramine (0.5  $\mu\text{g/ml}$ ), hexamethonium (20  $\mu\text{g/ml}$ ) and morphine (1  $\mu\text{g/ml}$ ). A three-minute response cycle is used for the agonist.
2. Activity of the solution containing acetylcholine disappears when boiled with N NaOH solution but not with weakly acidic solution.
3. Activity disappears rapidly when mixed with blood (due to its cholinesterase content) but much more slowly when the mixture contains eserine.

When it is almost certain that acetylcholine is the only substance present, the single most convenient method may be chosen for its assay. When this is not the case, at least two test objects must be used for any reliable result. Usually the blood pressure in anaesthetized cat, and either the frog rectus or the leech muscle are employed, the rectus giving more reproducible results, and eserinated leech muscle higher sensitivity. Effect of eserine on frog rectus is much less striking than in the case of the leech muscle; the usual sensitization being about 10-fold as against 1000-fold for leech muscle. The appropriate sensitizing (eserine) and antagonizing (atropine or curare) drugs should be used wherever applicable, and the alkali-inactivation procedure in every instance.

For testing extracts, the standard acetylcholine should always be prepared by Feldberg's method as follows: The standard acetylcholine solution instead of being made up in Locke's solution is mixed with a portion of the extract to be tested, which has previously been freed from acetylcholine by boiling with one-tenth volume of N NaOH for one minute and then neutralizing with N HCl. The dose of the test extract must represent the same amount of tissue as the dose of standard extract (acetylcholine-free) with which it is being compared.

*Frog rectus muscle.* The frog rectus muscle is put up in an organ bath and attached to a simple writing lever having tension 0.75 to 1 g initially reduced to 0.5 g at the

beginning of the assay. The preparation may be sensitized by adding neostigmine, which is superior to eserine as a sensitizing agent. A standard solution of acetylcholine is added to the bath and a slow contracture is recorded on the slow-moving drum for exactly 90 seconds. The drum is stopped and the bath fluid is replaced by fresh frog-Ringer solution. The lever is pressed gently to extend the muscle to its original length (i.e. back to the base line) or an extra 1 g load is used as a stretcher-weight for a few minutes. A dose interval of five minutes is followed throughout and a matching assay of the unknown carried out. The method is fairly sensitive (threshold concentration about  $10^{-8}$ , cf. leech muscle  $10^{-9}$ ) and accurate (error  $\pm 5\%$ , cf. leech muscle  $\pm 15$  to  $20\%$ ), and probably the most convenient and precise of the methods generally available.

*Cat blood pressure.* For details see Chapter 21.

### DETECTION AND ESTIMATION OF HISTAMINE

*Isolated guinea pig ileum.* The isolated ileum of guinea pig is the most sensitive and accurate test object for the assay of histamine. A piece of terminal ileum is put up in an isolated bath in Tyrode solution containing  $10^{-7}$ g/ml atropine sulphate. This eliminates or reduces contractile responses due to cholinergic agents, causes relaxation of the gut producing a fixed base line, and reduces or eliminates spontaneous contractile activity in the gut. All these effects contribute to the precision of the assay. Contractions of the gut to histamine are usually complete in 30 seconds or less; relaxation during rinsing or washing of the bath with two or three changes of Tyrode solution is almost complete in another 30 seconds. Hence, contractions for exactly 30 seconds are regularly obtained at intervals of one to one and half minutes.

The preparation usually gives a good response to histamine in a concentration of  $10^{-8}$  g/ml. A matching assay is carried out with the standard and the results expressed in terms of base.

*Identification of histamine in an extract.* If the extract is boiled with strong acid, the pharmacological activities of common contaminants like adenosine and substance P but not of histamine are destroyed. Treatment of the tissue with atropine ( $0.3 \mu\text{M}$ ) renders it insensitive to acetylcholine. It is rendered sensitive by adding propranolol ( $3 \mu\text{M}$ ) in the bathing fluid. It is not enough merely to demonstrate that an antihistamine abolishes the effect of the extract. A dose of the latter (mepyramine  $10^{-8}$  to  $10^{-7}$ ) sufficient to reduce but not to abolish the response of the gut to doses of histamine of the order of those used in the assay is added to the bath and washed out after two or three minutes. Alternate doses of the extract and approximate equivalent doses of histamine are then added into the bath at the usual time intervals. Identification of histamine can be regarded as established if the recovery of the response of the ileum to the extract and to histamine follows a similar time course (see Fig. 23.1).

*Cat or dog blood pressure.* For details see Chapter 21.

## DETECTION AND ESTIMATION OF ADRENALINE AND NORADRENALINE

*Isolated rabbit duodenum.* Both adrenaline and noradrenaline relax the smooth muscle of the rabbit intestine (both  $\beta$  and  $\alpha$  effects); the concentration of an unknown solution of which can be estimated by comparing its effect with that of a standard solution. The relative activity of adrenaline and noradrenaline varies in different pieces of intestine. Sometimes, adrenaline is more powerful, sometimes noradrenaline; hence an unknown solution of either of them must be compared with a known solution of the same substance. The duodenum (4 to 5 cm long) is put up in an organ bath (50 to 75 ml) and a matching assay is carried out with adrenaline or noradrenaline as a standard.

*Dog or spinal cat blood pressure.* For details see Chapter 21.

### ESTIMATION OF ADRENALINE AND NORADRENALINE IN A MIXTURE

*Rat uterus.* The best organ for the assay of adrenaline in presence of noradrenaline is the non-pregnant rat uterus on which noradrenaline has no effect. The most sensitive method of estimating adrenaline (sensitive to 1 ng/ml) is to determine its power to antagonize the contractile actions of acetylcholine on this preparation.

One horn of the uterus is put up in an organ bath. A contraction is obtained by adding acetylcholine (2  $\mu\text{g/ml}$ ) or carbachol (0.75  $\mu\text{g/ml}$ ) to the bath and washed after 30 seconds and the bath fluid changed twice when the muscle relaxes. After 2 minutes (i.e. 90 seconds after washing the first dose), a second dose of acetylcholine is given, and the whole cycle is repeated every 2 minutes until the muscle shows uniform contractions. A known amount of adrenaline (1 to 10 ng) is then added into the bath 30 seconds before a dose of acetylcholine, and the amount of reduction of the latter's effect noted. As soon as the inhibitory effect of adrenaline disappears and the acetylcholine effect completely recovered, a dose of the unknown adrenaline is tried so as to carry out a matching assay.

When the amount of adrenaline in the mixture has thus been determined (noradrenaline usually has only one-hundredth of the effect of adrenaline in this preparation that can be disregarded), the mixture (usually an extract) is injected intravenously into a spinal cat, and an equivalent amount of adrenaline in this mixture also injected for comparison. The rise of blood pressure caused by adrenaline will be less than that caused by the mixture. Noradrenaline is now added to this amount of adrenaline until the rise of pressure caused by the mixture is equal to the rise of pressure caused by the unknown mixture (extract). The amount of noradrenaline added is then equal to the amount present in the extract.

*Rat colon.* This tissue provides a sensitive test for noradrenaline which generally has slightly more effect than equal dose of adrenaline (ratio varying from 0.1 to 1). In combination with the rat uterus, this preparation can be used for assaying the

two catecholamines in a mixture but the error is large. The procedure is rather similar to that of rat uterus. The temperature of the bath containing de Jalon's solution is lowered until spontaneous contractions disappear (usually 25 to 27°C). If still troublesome, the spontaneous movements may sometimes be abolished by using a lower concentration of calcium chloride (0.003%). The dose of acetylcholine and of carbachol is lower than that for uterus (5 to 50 ng/ml).

### DETECTION AND ESTIMATION OF 5-HYDROXYTRYPTAMINE

Both rat uterus and stomach are highly sensitive to 5-hydroxytryptamine.

*Rat uterus.* Rats in natural oestrus may be selected. In a 5 ml bath as little as 10 ng 5-hydroxytryptamine can be detected. A contact for one and a half minutes and dose intervals of five minutes are used for the assay by this method.

*Rat stomach.* In a 5 ml bath as little as 1 ng 5-hydroxytryptamine can be detected. Hyoscine reduces sensitivity to acetylcholine. Histamine, bradykinin and adenosine compounds stimulate this preparation in microgram doses, while vasopressin stimulates only in moderately high doses. Sensitivity to inhibition by adrenaline and noradrenaline is high. Specific blockade of 5-HT is produced by methysergide.

### DETECTION AND ESTIMATION OF OXYTOCIN

*Rat uterus.* Rats are pretreated 36 to 48 hours earlier with 0.1 mg diethylstilboestrol intramuscularly. In general, calcium tends to increase both the spontaneous activity of the uterus and the sensitivity to drugs. Magnesium has the reverse effect, though there is some evidence that it can potentiate the effects of posterior pituitary hormones. De Jalon's solution, containing a quarter of the amount of calcium in Ringer-Locke solution, is used for the assay of oxytocin. The slight loss of sensitivity to oxytocin is more than offset by the lack of spontaneous activity. Other measures to control the erratic behaviour of this preparation are lowering of the bath temperature to 25 to 35°C, and of pH by aerating with oxygen containing 5% CO<sub>2</sub>. Doses of oxytocin are usually 0.1 to 3 milliunits per ml; contact for 30 seconds at intervals of five minutes.

### IDENTIFICATION AND ASSAY OF AN UNKNOWN SUBSTANCE

*Isolated guinea pig ileum.* A piece of terminal ileum of guinea pig is put up in an organ bath. Submaximal contractions are obtained with acetylcholine starting with a dose giving a bath concentration of 0.01 µg/ml (contact for 30 to 60 seconds) and then repeating every one and half to three minutes, if necessary, in 10-fold increasing doses. After obtaining two or three uniform contractions, the bath is washed and the test material added at a bath concentration of 1 µg/ml. The response, if any, is recorded for one minute, and then after washing the dose of acetylcholine repeated. If there is no response on its own, nor any change in the subsequent acetylcholine response, the dose of the test material is added repeatedly after each washing in 5



to 10-fold increasing doses up to a maximum of 1 mg/ml bath fluid. Thus, the material can be found to be inactive, or to have a stimulant effect, or antagonistic to acetylcholine.

If the test material has a stimulant action of its own, the nature of the compound and its site of action are determined. The response to a stimulant dose of the material is observed for one minute. Then a response to a matching dose of acetylcholine is obtained. A dose of atropine 0.01  $\mu\text{g/ml}$  bath fluid is now added, and after one minute the dose of acetylcholine repeated, and complete or partial inhibition of response observed. After repeated washings and complete recovery of acetylcholine response, the experiment is repeated with atropine followed by the test material. If the response remains unaffected, the contraction produced by the test material is probably not due to muscarinic action. The experiment is then performed in a similar fashion with matching dose of nicotine and blocked with hexamethonium 10  $\mu\text{g/ml}$ . If the response due to the test material but not due to nicotine remains unaltered, then the material lacks a ganglionic stimulant action. If the response is due to histamine-like action, it will be blocked by mepyramine 0.01  $\mu\text{g/ml}$  as will be the response of a matching dose of histamine. On the other hand, if it is due to 5-hydroxytryptamine-like activity, its response as well as that of 5-hydroxytryptamine will be antagonized by methysergide 0.5 ng/ml. If the response of the test material cannot be antagonized by any of the above means, it is probably a polypeptide having direct stimulant action on the smooth muscle.

The antagonistic activity of the test material can be studied by including three more substances (histamine 0.2  $\mu\text{g}$ , 5-hydroxytryptamine 0.2  $\mu\text{g}$  and nicotine 2  $\mu\text{g/ml}$ ) and studying them one after another as with acetylcholine. In this way, any antagonistic action of the test material against the four standard stimulants can be detected. Furthermore, from these tests a preferential or specific antagonism against a particular substance, or a nonspecific antagonism against all the four substances can be determined. For instance, if it is selective against acetylcholine, i.e. a dose which blocks the effect of acetylcholine and not the other three substances, then it is supposed to have a muscarinic-blocking action like atropine. Similarly, antihistaminic, antinicotinic and antiserotonin actions can be determined by using a specific concentration of the material. On the other hand, if all the four standard stimulants are blocked to about the same extent by a particular dose of the test material, it has either a direct smooth muscle depressant action like papaverine, or a sympathomimetic action like adrenaline or noradrenaline.

*Guinea pig vas deferens.* The papaverine-like and sympathomimetic actions are differentiated by testing the material on guinea pig vas deferens, a preparation that responds to adrenaline-like substance by contraction, but not to papaverine-like substance. If the test material has a stimulant action, a matching contraction is obtained with adrenaline (dose contact 45 seconds and interval five minutes). The sympathomimetic action of the material is confirmed further by almost equal blocking of both the responses by an adrenergic blocking drug. If the test material

## Identification and Estimation of Biologically Active Substances

has no stimulant action on this preparation but reduces the responses to acetylcholine and histamine, it has papaverine-like effect. This can be confirmed by administering matching doses of histamine and acetylcholine before and after an effective dose of the test material. Nonspecific antagonism like papaverine will be confirmed by observing almost equal antagonism of both the responses.

The interpretation of results obtained on isolated guinea pig ileum and vas deferens experiments are summarized in Table 23.1.

**Table 23.1**  
Interpretation of results obtained on guinea pig ileum and vas deferens

A. <i>If the compound stimulates ileum which is antagonized by :</i>	<i>Inference</i>
Atropine (0.1 $\mu$ g/ml.)	Muscarinic
Hexamethonium (100 $\mu$ g/ml.)	Ganglionic stimulant
Mepyramine (0.01 $\mu$ g/ml.)	Histaminic
Methysergide (0.5 ng/ml.)	Serotonin-like
None of the above	Direct smooth muscle stimulating biologically active polypeptides
B. <i>If the compound antagonizes the ileum stimulated by :</i>	
Acetylcholine	Atropine-like
Histamine	Antihistaminic
Serotonin	Antiserotonin
Nicotine or DMPP	Ganglionic blocking or local anaesthetic (differentiated by testing on cat nictitating membrane preparation, and for local anaesthetic activity in guineapig)
Nicotine and serotonin	Same as above
All the above	Papaverine-like or sympathomimetic
C. <i>If the compound stimulates vas deferens which is antagonized by:</i>	
Phenoxybenzamine or phentolamine	Sympathomimetic amines
D. <i>If the compound antagonizes vas deferens stimulated by :</i>	
Acetylcholine, histamine	Papaverine-like activity confirmed by equal antagonism of acetylcholine and histamine by the same dose of the compound

### IN VITRO SCREENING OF ACTIVE SUBSTANCES PRESENT IN BIOLOGICAL FLUIDS AND TISSUES

#### A. Tested on Guinea pig Ileum

##### Contraction:

- 5-Hydroxytryptamine
- Acetylcholine
- Angiotensin
- Bradykinin
- Histamine

##### Relaxation or no effect:

- Noradrenaline
- Adrenaline

- B. Tested on Rat Duodenum**  
*Contraction:*  
 5-Hydroxytryptamine  
 Acetylcholine  
 Angiotensin  
*Relaxation:*  
 Bradykinin  
*No effect:*  
 Histamine
- C. Tested on Rat Ascending Colon**  
*Contraction:*  
 5-Hydroxytryptamine  
 Acetylcholine  
 Histamine  
 Angiotensin

The identification of the compound is finally confirmed by the use of respective antagonists.

The sensitivity of different tissues to biologically active substances is presented in Table 23.2

**Table 23.2**  
 Sensitivity of different isolated preparations to some biologically active substances\*

Preparation	5-HT	ACh	AT	BK	NA	A	H
Rat ascending colon	$2.0 \times 10^{-7}$ (C)	$2.0 \times 10^{-8}$ (C)	$2.0 \times 10^{-9}$ (C)	$2.0 \times 10^{-7}$ (R)	$2.0 \times 10^{-7}$ (R)	$2.0 \times 10^{-7}$ (R)	$10^{-8}$ (C)
Rat stomach strip	$10^{-8}$ (C)	$2.0 \times 10^{-8}$ (C)	$2.0 \times 10^{-8}$ (C)	$2.0 \times 10^{-8}$ (C or R)	$2.0 \times 10^{-7}$ (R)	$2.0 \times 10^{-7}$ (R)	0
Rat duodenum	$2.0 \times 10^{-8}$ (C)	$2.0 \times 10^{-8}$ (C)	$2.0 \times 10^{-7}$ (C)	$2.0 \times 10^{-8}$ (R)	$2.0 \times 10^{-7}$ (R)	$2.0 \times 10^{-7}$ (R)	0
Guinea-pig ileum	$2.0 \times 10^{-8}$ (C)	$2.0 \times 10^{-8}$ (C)	$2.0 \times 10^{-8}$ (C)	$2.0 \times 10^{-7}$ (C)	$2.0 \times 10^{-7}$ (R)	$2.0 \times 10^{-7}$ (R)	$10^{-8}$ (C)

Values are g/ml bath fluid  
 C = Contraction, R = Relaxation, 0 = No response  
 \* Modified after Regoli and Vane, 1964

**POLYPEPTIDES THAT STIMULATE SMOOTH MUSCLES\***

The polypeptides like oxytocin, vasopressin, angiotensin, bradykinin and substance P have the common property of stimulating smooth muscles. These may, however, be differentiated by one or more of the following tests.

*Isolated guinea pig ileum.* Excepting oxytocin, all stimulate this preparation to a varying extent. Bradykinin and angiotensin are about 30 times more sensitive than vasopressin, and about five times more than substance P on this tissue.

\* Schacter and Morley, 1964

## Identification and Estimation of Biologically Active Substances

*Isolated rat uterus.* Substance P is least sensitive in this preparation in producing contraction, being 10 times less than vasopressin, 50 times less than oxytocin and angiotensin, and 100 times less than bradykinin.

*Isolated rat colon.* Substance P is most sensitive being about 700 times more than oxytocin, vasopressin and bradykinin, and about 350 times more than angiotensin in producing contraction.

*Isolated rat duodenum.* Except substance P that contracts, all others produce relaxation. Bradykinin is about 1000 times more sensitive than vasopressin, and about 3000 times more than oxytocin as relaxant.

*Blood pressure in anaesthetized animals.* Vasopressin, oxytocin and angiotensin produce a pressor effect, while bradykinin and substance P a depressor effect on rat or rabbit blood pressure. In rabbit, substance P is 100 times more sensitive than bradykinin, while in rat it is only 20 times more sensitive as a depressor agent. Angiotensin and vasopressin are about 35 times more potent than oxytocin as a pressor agent in rat.

*Rat antidiuresis.* Vasopressin is several thousand times more sensitive than all others.

*Bronchoconstriction in anaesthetized guinea pig.* Bradykinin is about 40 times more sensitive than angiotensin, oxytocin and vasopressin, and one to four times more sensitive than substance P in producing bronchoconstriction.

The effect due to bradykinin is specifically antagonized by salicylates and related drugs.

*Rabbit milk ejection.* Oxytocin is highly sensitive being several thousand times more effective than substance P, angiotensin and bradykinin, and 10 times more than vasopressin.

*Inactivation by enzymes.* All are inactivated by chymotrypsin; substance P, angiotensin and vasopressin are also inactivated by trypsin, and substance P and angiotensin in addition by pepsin.

### ASSAY METHODS OF POLYPEPTIDES

<i>Polypeptide</i>	<i>Preparation</i>
Oxytocin	Rat isolated uterus (stilboestrol treated)
Vasopressin	Blood pressure in anaesthetized rat (pretreated 24-hour earlier with phenoxybenzamine)
Angiotensin	Guinea pig isolated ileum Rat ascending colon Blood Pressure in anaesthetized dog or rat (treated with phenoxybenzamine and atropine)
Bradykinin	Rat isolated duodenum Guinea pig isolated ileum
Substance P	Guinea pig isolated ileum (treated with atropine, mepyramine and methysergide)

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# Some Common Evaluation Techniques

## EVALUATION OF ANALGESIC AGENTS

In general three types of stimuli – physical, thermal and chemical are employed in mice or rats for evaluation of analgesic property of a compound. Since false positive results are sometimes obtained with these tests, a *discoordination* test is carried out to exclude such possibilities. The animals are placed on a slowly rotating drum covered with a wire-mesh; the animals that fall off are considered as discoordinated and they are excluded from the experiment.

The analgesic tests are usually carried out as follows.

### PHYSICAL STIMULUS

#### Tail-Clip Method\*

In this method, an artery clip with thin rubber sleeves is applied to the base of the mouse's tail for 30 seconds. Control mice make continuous efforts to dislodge the clip by biting it. The pressure exerted by the clip is so adjusted that it is just sufficient to make all control mice to respond. Analgesics make the mice indifferent to the clip. A group of mice is injected subcutaneously with a dose of the test substance and 30 minutes later the clip is applied. The percentage of mice in a group that fails to respond is calculated. By employing graded doses the ED50 is found out. The analgesic tested by this method involves the higher centres.

### THERMAL STIMULUS

#### Hot-Plate Method\*\*

Mice or rats are placed on a hot plate maintained at 55°C. The reaction time is that between placing the animals on the hot plate and licking of the fore or hind paws. A cut off time of 30 seconds is followed to avoid any thermal injury to the paws. The mean increase in the reaction time is plotted against time after drug administration, and the area under the time-response curve is calculated either graphically or planimetrically. By using different doses, a dose-reaction time curve can be plotted and ED50 value worked out.

\* Bianchi and Franceschini, 1954

\*\* Eddy and Leimbach, 1953

**Tail-Flick Response\***

Mice or rats are held in suitable restrainer with the tail protruding out. Radiant heat is applied over the tail on a single spot with the help of a suitable device. The time taken by the animal to withdraw (flick) the tail is taken as the reaction time. In this method the animals are selected by preliminary screening. Those showing variation of more than one second between two reaction times at 15 minute interval or more than three seconds from the group mean are discarded. The time-response curves may be plotted or ED50 values calculated.

**Tail-Immersion Test**

Mice or rats are held in position in a suitable restrainer with the tail protruding out. The tail up to 5 cm is dipped in a beaker of water at 55°C. The time taken to withdraw the tail clearly out of water is taken as the reaction time.

**CHEMICAL STIMULUS****Writhing Test**

Intraperitoneal injection of phenylquinone (0.25 ml of 0.02% aqueous solution) or acetic acid (1 ml/100 g body weight of 0.6% acetic acid) in mice produce within 3 to 10 minutes a *writhing or stretching syndrome* characterized by a wave of contractions of the abdominal musculature followed by extension of the hind limbs. These chemicals, however, tend to produce irritation and cannot be repeated at shorter intervals to study the tolerance development of the drug under investigation. Further, the test may give false positive results.

**Writhing Induced by 4% NaCl Solution\*\***

The writhing test with 4% NaCl in rats is a sensitive and specific test for predicting analgesic activity of a compound in man. It is also useful for examining changes in analgesic activity of drugs during chronic (repeated) administration. Male rats (150 ± 10 g) are injected intraperitoneally with 1 ml/kg of 4% NaCl. Animals that do not exhibit writhing within 30 seconds are discarded. The onset of writhing is much quicker (30 sec) than that with phenylquinone or acetic acid, and lasts for a shorter period (about 3 min). The drug under investigation is administered subcutaneously 15 to 20 minutes before administering NaCl solution. The animals showing no response are defined as analgesic positive. The percentage protection at each dose level is calculated for each group of 10 to 20 animals, and the ED50 values are calculated from the dose-response curves.

**Writhing Induced by Aconitine\*\*\***

This test is found to be selective for aspirin-like analgesics. Each mouse is

\* D'Amour and Smith, 1941

\*\* Fukawa *et al.* 1980

\*\*\* Bhalla and Bhargava, 1980

injected intra-peritoneally with 2  $\mu\text{g}$  of aconitine. The writhing appears within two minutes and lasts for about 60 minutes.

### DETERMINATION OF $\text{pA}_2$ VALUE OF ANALGESIC – ANTAGONIST COMBINATION\*

The responses of the analgesic in the absence and in the presence of different doses of antagonist (say, morphine-naloxone) are determined in mice. The dose ratios ( $\text{ED}_{50}$  with antagonist /  $\text{ED}_{50}$  without antagonist) thus obtained are plotted against the negative log molar dose of antagonist (Schild plot – see Chapter 19) and the apparent  $\text{pA}_2$  value is calculated. Comparison of the  $\text{pA}_2$  values with different analgesic-antagonist combinations gives an idea of the types of receptors involved. An almost identical  $\text{pA}_2$  value with different types of analgesic tests (tail-flick, hot plate and writhing) will indicate the involvement of identical receptors.

### EVALUATION OF ANTI-INFLAMMATORY AGENTS

The main shortcoming in the screening of anti-inflammatory drugs for use in human arthritic disease is the lack of suitable animal model. Moreover, the aetiological agents that may trigger off the various connective tissue diseases are not known as yet. Anti-inflammatory agents have been evaluated by studying inflammatory responses produced in the animals by injecting foreign or noxious agents. These responses mostly comprise of the development of oedema and/or the formation of exudate and granuloma. Agents that suppress any of these are designated as *anti-inflammatory*. The term *anti-inflammatory* has so many connotations that multiple assays are required to affirm this property in any chemical compound.

The complete process of inflammation generally consists of three phases (i) dilatation and increased permeability of small blood vessels resulting in oedema and swelling, (ii) emigration of leucocytes from venules and capillaries, cellular infiltration, and a general mopping up reaction, and (iii) proliferation of fibroblasts and synthesis of new connective tissue to repair the injury.

A number of mediators have been identified that initiate the early development (first phase) of certain experimentally induced inflammatory processes. These are considered to be released in a sequential manner. Thus, there is an initial release of histamine and 5-hydroxytryptamine (5-HT) producing an increased vascular permeability followed by release of kinins further contributing to the increased vascular permeability and finally, the prostaglandins and slow reacting substance (SRS) are released to maintain the increased vascular permeability produced by histamine, 5-HT and kinins (Smith *et al.* 1974).

The biochemical events accompanying the second phase are not well understood. Many factors are implicated as the regulators of phagocytosis including

\* Hyashi and Takemori, 1971



calcium chemotoxin, leucocyte promoting factor, complement factor, etc. These chemotactic factors play a major role in the process of leucocytic migration. Polymorphonuclear leucocytes predominate in the cellular infiltrate during the early stages; subsequently, their number diminishes and granular mononuclear leucocytes (monocytes) become the predominating leucocyte in the inflamed tissue (Vinegar *et al.* 1973).

As the exudative phase of inflammation subsides, the initial stages of the reparative or third phase are set in motion. The fibroblast, which is the dominant cell in the wounded zone, first proliferates then synthesizes extracellular material including new collagen fibres and acidic mucopolysaccharides, which are laid down to form the new connective tissue matrix (Whitehouse, 1965).

On the basis of the different aspects of inflammation, the following experimental models are generally selected to investigate the anti-inflammatory activity of a chemical compound.

#### Rat Paw Oedema

Oedema represents the early phase of inflammation. Carrageenan-induced paw oedema (Winter *et al.* 1962) is the simplest and most widely used model for studying the anti-inflammatory activity of new compounds. Similarly, the paw oedema induced by histamine, 5-HT, bradykinin, dextran, hyaluronidase and prostaglandin  $E_1$  have been used for studying the antagonism to these mediators (Ghosh *et al.* 1963; Ghosh and Singh, 1974; Parmar and Ghosh, 1978). These agents can be injected in a 0.1 ml volume of suitable concentration in sterile saline into the subplantar tissue of the rat hind foot, and the paw volume can be measured immediately, and then at pre-determined intervals by the plethysmometric method of Singh and Ghosh (1968).

#### Granuloma Pouch

Granuloma represents the exudative and proliferative phases of inflammation. The cotton pellet method described by Meir *et al.* (1950), and the croton oil method described by Selye (1953) have been most widely used for producing granulomas. Fukuhara and Tsurufuji (1969) have described a different method of producing granuloma pouch using 4 ml of 2% (w/v) carrageenan in sterile normal saline. It has certain advantages over the classical methods as follows. Unlike the croton oil granuloma, the necrosis of pouch-wall does not occur in this model. The granulation tissue formed can be clearly separated from the body and the exudate can be completely recovered and measured correctly. In the cotton pellet method, the inflammation is measured by weighing the capsular granuloma together with the cotton pellet, which is often contaminated with the exudate and pus; hence it often gives inconsistent results. In the carrageenan method, all the three desired parameters, i.e. volume of exudate, weight of the pouch wall and the accumulation of collagen in the granuloma can be satisfactorily studied.

## Some Common Evaluation Techniques

### Experimental Pleurisy

Pleurisy induced by the intrapleural injections of irritants, like turpentine and carrageenan in the rat, permits the experimental production that is typically exudative. This has been widely used for studying the effect of drugs on the migration of leucocytes (Ford-Hutchinson *et al.* 1975).

### Adjuvant-Induced Arthritis

Adjuvant-induced arthritis is the only experimental model, which has often been considered as parallel to the human arthritic disease more closely than any other laboratory model (Newbould, 1963; Sofia *et al.* 1975). Hence, it is considered desirable to include this model in any inflammatory study to be of predictive value for potential clinical use. The arthritic syndrome is induced by the intradermal injection of 0.05 ml of a fine emulsion of dead tubercle bacilli (5 mg/ml) into the plantar surface of right hind foot. The following parameters are noted in the control and in the treated groups throughout the period of 14 days: (i) paw oedema, (ii) the severity of secondary lesions, and (iii) body weight changes.

## EVALUATION OF COMPOUNDS ON GASTROINTESTINAL FUNCTIONS

### ASSAY OF ANTI-ULCER AGENTS

Several standard animal models are used for the evaluation of various agents for their possible gastric anti-ulcer properties.

### Pylorus Ligated (Shay) Rat\*

Albino rats weighing 140 to 165 g are housed in individual cages and fasted (water allowed) for 48 hours prior to pyloric ligation, care being taken to avoid coprophagy. Under light ether anaesthesia the abdomen is opened by a small midline incision below the xiphoid process; pyloric portion of the stomach is slightly lifted out and ligated avoiding traction to the pylorus or damage to its blood supply. The stomach is replaced carefully and the abdominal wall closed by interrupted sutures. The drugs are administered subcutaneously once daily for two days prior to and immediately after pyloric ligation. They are deprived of both food and water during the postoperative period and are sacrificed at the end of 19 hours after operation. Stomach is dissected out and the contents subjected to analysis for pH and for free and total acidity. The stomach is then cut open along the greater curvature and the inner surface is examined for any ulceration. The degree of ulceration is graded from zero to five, depending on the size and severity of ulcers as described by Barret *et al.* (1953).

\* Shay *et al.*, 1945

**Restraint Ulcer in Rat\***

Rat after fasting for 36 to 40 hours (water allowed) is lightly anaesthetized with ether and placed on a piece of galvanized steel window-screen of appropriate size. The screen is moulded round the animal and held in place with wire staples. The limbs are held together in pairs and tightened with adhesive tapes so that the animal cannot move. The drugs are administered either orally or subcutaneously once daily for two days prior to and 30 minutes before subjecting the animal to stress. At the end of 24-hour period the animal is removed from the screen, sacrificed, and the degree of ulceration assessed.

**Drug Induced Gastric Mucosal Damage in Rat \*\***

Gastric ulceration in rats is induced by drugs, and the ability of several agents either to protect against or to aggravate this ulceration is observed. Generally, the compounds under investigation are administered once daily for two days prior to and 30 minutes before administration of the ulcerogenic agent. The stomach of the sacrificed animal is examined for the presence of mucosal lesions. The incidence and grading of ulcers are done according to the method described by Wilhelmi and Menasse-Gdynia (1972). Necro-haemorrhagic spots greater than 2 mm diameter are taken as ulcers. These are graded as follows: 0.5 – minute, sporadic, punctuate lesions; 1 – several small lesions; 2 – one large extensive lesion, or multiple moderate sized lesions; and 3 – several large lesions.

The following drugs are routinely used as ulcerogenic agents.

*Aspirin.* This is suspended in 1% carboxymethylcellulose and administered orally through an intragastric polythene tubing (gavage) in a dose of 200 mg/kg; four hours later the animals are sacrificed.

*Phenylbutazone.* As a gavage in a dose of 100 mg/kg. Two doses are given at an interval of 15 hours; six hours after the second dose the animals are sacrificed.

*Indomethacin and ibuprofen.* As a gavage indomethacin is given in two doses of 10 mg/kg, while ibuprofen is given in two doses of 200 mg/kg at 15-hour interval; six hours after the second dose the animals are sacrificed.

*Reserpine.* A single injection of 5 mg/kg is given intramuscularly; 24 hours later the animals are sacrificed.

**Histamine Induced Gastric Ulceration in Guinea pig\*\*\***

Male guinea pigs weighing 300 to 450 g are fasted for 48 hours (water allowed). Ulceration is induced by injecting 1 ml of histamine acid phosphate soln. (50 mg base) intraperitoneally. Promethazine hydrochloride 5 mg is injected intraperitoneally 15 minutes before and 15 minutes after histamine to protect the animals against histamine toxicity. The drug under investigation is given orally once daily for two days 30 to 45 minutes before histamine injection. The animals

\* Brodie and Hanson, 1960 \*\*Hemmati *et al.* 1973; Gupta *et al.* 1974 \*\*\*Zaidi and Mukerji, 1958

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are sacrificed on the second day four hours after histamine administration, and the stomach is dissected out. The gastric contents are subjected to analysis; the stomach is cut open and the degree of ulceration is graded depending on the size and intensity of the ulcers as described by Barret *et al.* 1953.

### ASSAY OF ANTI-SECRETORY AGENTS

#### Continuous Recording of Gastric Acid Secretion in Rat\*

In this method, several doses of drugs can be given in the same animal for a quantitative assay purpose. Male rats weighing 180 g and above are starved overnight and anaesthetized with 25% urethane solution (0.6 ml/100 g i.m.). Trachea and external jugular vein are cannulated. By a midline incision on the abdomen, the pyloric end of stomach is exposed and cannulated. A flexible polythene tube is passed down the oesophagus and tied in the cervical region. The stomach is washed out thoroughly by passing distilled water through the tube and allowing it to come out of the pyloric cannula. The stomach is then perfused continuously at a uniform rate (1 ml per minute) with N/4000 NaOH soln. either by using a gravity feed system using a high pressure and high resistance to control flow (Mariotte bottle), or by using a peristaltic pump. The concentration of NaOH may be adjusted so that the perfusate under basal condition has a pH 6 to 6.5. The perfusate after emerging out of the pylorus bathes a microflow glass electrode connected to a direct-reading pH meter, which in turn is connected to an ink recorder. Changes in the acid secretion are recorded as changes in pH in response to secretagogues like histamine, carbachol, etc. administered intravenously. The drug under investigation is injected prior to each dose of a secretagogue and any inhibition or potentiation of the effect is recorded.

### ASSAY OF DRUGS AFFECTING INTESTINAL MOTILITY

#### Charcoal Meal Test in Mice\*\*

Adult mice are given a charcoal meal (animal charcoal 12 g, tragacanth 2 g, water 130 ml) by gavage and killed 15 minutes later. The abdomen is opened; the small intestine from pylorus to the ileocaecal junction is dissected out and its length is measured. The distance that the charcoal meal has traveled is also measured and expressed as a percentage of the total length of the small intestine. The drugs are administered by gavage 45 minutes before the charcoal meal and intestinal motility determined by assessing the rate of passage of the charcoal meal.

#### Rat Treated with Castor Oil\*\*\*

Rats were administered either saline (control) or test substance orally 30 min

\* Ghosh and Schild, 1958. \*\* Macht and Barbara-Gose, 1931 \*\*\* Vischer and Casals-Stenzel, 1983

before oral administration of castor oil (2 ml per rat) and charcoal meal (1 ml/100 g). The rats are killed by exsanguination 30 min after castor oil and charcoal meal administration. The abdomen is opened, the small intestine from the pylorus up to the caecum is carefully removed and gently stretched. The total length of the intestine and the distance travelled by the black marker is measured. Intestinal transit is expressed as a percentage of the length traversed by the charcoal marker to the total length of the small intestine.

**Intraluminal Fluid Accumulation in Rat\***

Saline or test substance is administered orally to 18 h fasted rats 30 min before oral administration of castor oil (2 ml per rat). After a further period of 30 min the rats are killed by exsanguination. The pyloric and the caecal ends of the small intestine are tied, the intervening portion of the intestine is removed; the intestinal content is then collected and its volume measured.

**Normal Defaecation in Mice\*\***

Mice are placed individually in polythene cages with clean filter paper at the bottom. The saline or the test substance is administered orally; the number of faeces are counted at 2 hourly interval for 4 h; and the percent reduction in the treated group is compared with the control.

**Castor Oil Induced Diarrhoea in Mice†**

Mice are fasted for 12 h before the experiment. The test substance in increasing concentration or saline (control) are given orally 30 min before oral administration of castor oil (0.2 ml /mouse). The mice are then placed individually in polythene cages over clean filter paper. Two hours later the mice are scored for copious (++) , mild (+), or lack of diarrhoea (0) (Piercey and Ruwart, 1979). The activity score is calculated by taking the sum of the number of '+' mice and twice the number of '++' mice. Thus for a group of six mice the maximum score is 12 indicating severe diarrhoea. A score of zero indicates no diarrhoea.

**EVALUATION OF HEPATOPROTECTIVE AGENTS**

**Carbon Tetrachloride Induced Hepatotoxicity in Rats**

Carbon tetrachloride (CCl<sub>4</sub>), a potent hepatotoxin when introduced into the body is metabolized to CCl<sub>3</sub>, a free radical that binds to lipoprotein leading to peroxidation of lipids of the endoplasmic reticulum in liver. The cytoplasmic enzymes like glutamate pyruvate transaminase (GPT), glutamate oxaloacetate transaminase (GOT), etc. are released into the serum as a result of hepatocellular damage. An increase of these enzymes in serum serves as reliable indices in the

\* Robert et al., 1976

\*\* Melo et al., 1988

† Piercey and Ruwart, 1979

## Some Common Evaluation Techniques

assessment of the degree of hepatic damage. Decrease in these values by a compound indicates its hepatoprotective activity.

Liver toxicity is induced in a group of rats by subcutaneous injection of  $\text{CCl}_4$  suspended in liquid paraffin (1:2 v/v) in a dose of 1 ml/kg on alternate days for one week. The test substance is administered orally in another group of rats along with the administration of  $\text{CCl}_4$  on alternate days for one week. On the eighth day the blood is collected from retrobulbar plexus of the anaesthetized animals. The serum is separated after coagulating the blood at  $37^\circ\text{C}$  for 30 min and centrifuging, and estimated for GOT, GPT, alkaline phosphatase (ALP), acid phosphatase (ACP) and bilirubin. The values from the two groups are compared, and if the values in the treated group are significantly lower than those of the untreated group it suggests hepatoprotective effect of the test substance.

### Barbiturate Induced Sleeping Time in Mice

The hepatoprotective activity is studied in pentothal sodium induced sleeping time in mice treated with  $\text{CCl}_4$ . Liver is the primary site for biotransformation of pentothal sodium, an ultra-short acting barbiturate. Thus, its damage by  $\text{CCl}_4$  leads to an increase in the sleeping time in mice following pentothal sodium.

Sleeping time is measured in a group of mice injected with pentothal sodium 25 mg/kg i.p. In the second group,  $\text{CCl}_4$  1ml/kg i.p is administered two hours prior to pentothal sodium administration; the sleeping time is prolonged in this group. Test substance is administered prior to the  $\text{CCl}_4$  injection followed by pentothal sodium in the third group, and the sleeping time is measured. Any shortening in the sleeping time will indicate the hepatoprotective effect of the test substance.

## SCREENING OF ANTIFERTILITY AGENTS

A screening programme should clearly demonstrate the potentiality of a compound to produce temporary and fully reversible sterility in laboratory animals. The initial routine fertility tests may be carried out in both sexes followed by special tests to determine the mechanism of action. Rats are commonly used although mice, rabbits and monkeys may also be employed for the purpose.

### TESTS IN THE FEMALE RAT

Antifertility action in the female may result from the following: (i) inhibition of ovulation, (ii) prevention of fertilization, (iii) interference with the transport and/or implantation of the fertilized ovum, and (iv) destruction and resorption of the early implanted embryos.

The following tests are usually carried out for screening of antifertility drugs.

*Cohabitation test.* Female rats of established fertility (having produced two consecutive litters) are examined by vaginal smear for seven consecutive days for the presence of normal oestrous cycle. After administration of the test compound or the solvent the females are paired with males of proven fertility. The females

are examined for the following: (a) sign of mating as indicated by the presence of sperm in the vagina; prolongation of the average time from pairing to the first insemination is indicative of antifertility effect, (b) oestrous cycle changes by vaginal smear examinations, (c) inspection of uterus for number of implants, (d) number of litters at birth, and (e) resorption of foetuses revealed by the differences between (c) and (d).

*Ovarian weight in unilateral ovariectomized rat.* The ovarian weight increases in control animal 7-14 days after removal of the other ovary. A decrease in the ovarian weight in the treated animal compared to the control will indicate an inhibition of ovulation through suppression of follicular stimulating hormone. Ovarian histology may show corpus luteum, etc.

*Changes in the uterine weight.* Any change in uterine weight will indicate anti-oestrogenic action.

*Deciduomata.* The development of deciduoma in the endometrium will indicate progestational action.

#### TESTS IN THE MALE RAT

In the male, there are two possible mechanisms by which an antifertility agent may act: (i) suppression of spermatogenesis at any of the stages resulting in sterility associated with oligospermia or aspermia, and (ii) a qualitative change in spermatozoa rendering them nonfunctional.

Routine testing of male fertility is most conveniently carried out in rats, since tests extending over 12 weeks after treatment cover possible effects on any stage of spermatogenesis normally of 9 weeks duration. Any qualitative or quantitative change in sperm collected from the base of the epididymis is also studied.

*Cohabitation test.* Treated males are mated each with two females of known fertility. The females are examined for the following: (a) sign of mating as indicated by the presence of sperm in the vagina, (b) calculation of the date of insemination from the date of birth (gestation period approximately 21 days) permits an estimate of the duration of sterility, (c) pseudopregnancy (presence of leucocytes in vaginal smear for 10 to 14 days) is indicative of aspermic copulation, and (d) normal oestrous cycle is an indication of failure to mate due to the lack of libido.

#### EVALUATION OF ANTIDIABETIC AGENTS

Alloxan (mesoxalylurea) produces hyperglycaemia and glycosuria in most of the experimental animals except the guinea pig. Free radicals are involved in the production of DNA lesions by alloxan, which generate oxidized DNA bases with a preference for purines, thus contributing to their alkylating property. Streptozotocin (STZ), a glucose analogue, was originally isolated from the cultures of *Streptomyces achromogenes* by Herr *et al.* (1960). The glucose transporter 2 (GLUT2), which mediates glucose uptake into  $\beta$ -cells of pancreas also mediates the cellular uptake

of STZ. Reaching the pancreatic  $\beta$ -cells STZ causes fragmentation of DNA through formation of free alkylating radicals leading to reduction in the cellular levels of nucleotides and related compounds, particularly NAD<sup>+</sup>, which causes a rapid necrosis of  $\beta$ -cells. The classical rodent models (alloxan or STZ) represent type-I (IDDM) diabetes only. The streptozotocin-spontaneously hypertensive (STZ-SHR) rat and obese Zucker rat are examples of animal models where diabetes is associated with hypertension (van Zwieten 1999). The diabetogenic activity of STZ was first reported by Rakieta *et al.* (1963). It is now widely used to induce experimental diabetes in various laboratory animals.

For doses and routes of administration of alloxan and STZ in different species, refer to the "Guide to drug doses in laboratory animals" under Appendix.

Depending on the age of the rat, and the dose and route of administration of STZ, hyperglycaemic models of both type-I and type-II diabetes can be produced.

*Type-I diabetes:* Male adult rats are administered STZ (dissolved in citrate buffer) 40 mg/kg intravenously into the tail vein; after 14 days the blood glucose level is checked. The animals displaying more than 200-300 mg% of blood glucose are selected for the study. The substance under investigation is administered in predetermined doses and duration, and the blood glucose level in each animal is estimated at weekly intervals.

*Type-II diabetes:* Two-days old pups of rats are administered STZ 90 mg/kg intraperitoneally. The pups develop high blood glucose levels during their adulthood resembling type-II diabetes. The test substance is administered in predetermined doses and duration, and the blood glucose level estimated before and after the administration of the test substance.

Blood samples (0.05 ml) are obtained for glucose estimation either through the tail vein or by retro-orbital puncture using capillary tubes.

### EVALUATION OF ANTIPARKINSONIAN AGENTS

Since parkinsonism results from hypoactivity of the dopaminergic system or hyperactivity of the cholinergic system, either dopamine inhibitors or cholinomimetics are employed in the production of animal models for the evaluation of antiparkinsonian agents.

#### Oxotremorine Model

Oxotremorine [1-(2-oxo-1-pyrrolidinyl)-4-(1-pyrrolidinyl)-2-butynyl], a muscarinic agonist, is widely used to produce tremor in experimental animals similar to that of parkinsonism. The mouse is the most preferred model where oxotremorine 1-5 mg/kg i.p. produce tremor within 5 min, and the effect lasts for about 30 min (Fukuzaki *et al.* 2000). The test agent is administered 30 min before oxotremorine; after 10, 20 and 30 min, the tremor is scored in the individual mouse following a rating scale of 0 to 3 (0 – no tremor, 1 – occasional isolated twitches, 2 –



moderate or intermittent tremor with short period of quiescence, and 3 - pronounced continuous tremor). The tremor can also be measured objectively in a quantitative fashion by using a 'tremor monitor'.

#### LON - 954 Model

LON - 954 [N-carbamoyl-2-(2,6-dichlorophenyl) acetamide hydrochloride], a dopamine inhibitor and a tremorogen, produces a reproducible dose-dependent tremor in mouse at rest with an oral dose of 5-100 mg/kg. The tremor is rapid in onset, of short duration and of constant frequency without any sign of akinesia, muscle rigidity or antinociceptive activity.

#### MPTP Model

MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), a neurotoxin, produces neurochemical, histopathological and behavioural changes in animals resembling very closely the Parkinson's disease. Mice are administered MPTP 30 mg/kg i.p. twice a day for consecutive five days. One week after the last dose of MPTP, the above mentioned changes are observed (Ogawa *et al.* 1985).

### EVALUATION OF ANTICARCINOGENIC AGENTS

Some of the common animal models for the evaluation of anticarcinogenic agents are presented below :

#### Ehrlich Ascites Carcinoma (EAC)

Ehrlich ascites carcinoma cells in mouse are maintained by weekly intraperitoneal inoculation of  $10^6$  cells per mouse. Groups of Balb-C mice are inoculated i.p. with  $2-3 \times 10^5$  cells per mouse on day 0. Substances under investigation are administered either orally or intraperitoneally 24 hr after the inoculation, and continued for the duration of the study as per plan. Control mice are treated with equal volume of 0.9% saline. Median survival time (MST) for each group is noted. The viability of the cells are judged by the trypan blue test; the tumour cells are collected by repeated intraperitoneal wash with 0.9% saline, and viable tumour cell count is done using haemocytometer. Increase in the median survival time (MST), reduction of the viable cancer cells, and of the efficacy of the harvested cells to produce tumour are taken as the criteria for any anti-carcinogenic property of the substance under study (Sur and Ganguly, 1994).

#### Solid Tumour

Solid tumour is induced in Balb-C mice by subcutaneous injection of 3-methylcholanthrene (3-MC) 0.5 mg in castor oil into the calf muscle on day 1 and on day 7. The test substance is injected i.p. 3 days prior to 3-MC administration and continued as per the predetermined time schedule. The animals are killed by

## Some Common Evaluation Techniques

decapitation on the 6<sup>th</sup>, 8<sup>th</sup>, and 11<sup>th</sup> week after the last injection of 3-MC. The tumour is isolated and weighed, blood is collected from the retro-orbital plexus of the animals, and haematological and biochemical parameters are studied both for the control and for the treated groups of animals and results compared (Chaudhuri *et al.* 1998)

### Colon Cancer

Colon cancer is produced in adult rats (10–13 week old) by injecting azoxymethane (15 mg/kg) s.c. repeated after one week. The substances under study are administered i.p. for the predetermined time. At the end of 8 weeks all rats are killed by decapitation; the whole intestine from duodenum to anus is removed, slit open and flushed with normal saline. Neoplastic growths are counted along the entire length of the intestine. The colon is fixed in formalin saline and stained with 0.5% methylene blue to detect any pre-neoplastic lesions or cryptic foci in the tissue (Belobrajdic *et al.* 2003).

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# Toxicity Studies

It is essential to use at least two species (usually a rodent and a non-rodent) in the evaluation of the potential toxicity of a drug because species differ in their responses to toxic agents. It is also unwise to use a homogenous strain (inbred strain) in toxicity tests, and the aim should be to discover new and unexpected effects of a drug in animals of wider variability like random bred animals. A drug effect that is seen both in the rat and in the dog probably involves a common physiologic mechanism that is likely to be present in the human, whereas an effect seen only in one of the two species indicates that the same is peculiar to that species, and is less likely to be present in the third species. For instance, a toxic effect observed only in rats or dogs would indicate its probability of occurring in about 25 per cent in cases of man; while an effect observed in both rats and dogs would indicate a probability of 80 per cent.

The following tests are performed on laboratory animals for detection of toxicity of a compound.

*Acute toxicity test (single dose).* Test in which a single dose of the drug is used in each animal on one occasion only for the determination of gross behaviour (Table 25.1) and LD50 or median lethal dose (MLD), i.e. the dose which will kill 50 per cent of the animals of a particular species. LD50 value is determined in a 24-hour test using two species, one rodent (mice or rats) and one non-rodent (usually rabbit), and two routes of administration, one by intended route to be used subsequently.

Table 25.1  
Signs recorded during acute toxicity studies

Increased motor activity	Decreased motor activity	Salivation:
Tremors	Ataxia	viscid
Clonic convulsions	Sedation	watery
Tonic extension	Muscle relaxation	Writhing
Straub reaction	Hypnosis	Respiration:
Pilo-erection	Analgesia	depression
Muscle spasm	Anaesthesia	stimulation
Catatonía	Arching and rolling	failure
Spasticity	Ptosis	Skin colour:
Opisthotonus	Lacrimation	blanching
Hyperesthesia	Exophthalmos	cyanosis
Loss of righting reflex	Diarrhoea	vasodilatation

*Subacute toxicity test (daily doses).* Test in which animals (usually rats and dogs) are dosed daily, starting at around expected therapeutic level and increasing stepwise every two to three days until toxic signs are observed. Hematological

and biochemical monitoring is carried out, and blood level of the compound checked to ensure its absorption. The animals are maintained at the maximum tolerated dose for a period of two to three weeks to allow development of any pathological changes, and then killed and subjected to full pathological and histological examinations. The purpose of this test is to determine the maximum tolerated dose, and to indicate the nature of toxic reactions, so that suitable chronic toxicity studies can be designed to evaluate fully the toxic potential of the compound. Daily or weekly body weight changes are recorded. Inhibition of growth rate in young male rats (21 days old weighing 40 to 50 g) is a simple and sensitive test for evidence of some toxicities of the compound.

*Chronic toxicity test* (daily doses). Tests in which two species, one rodent (usually rat) and one non-rodent (usually dog or a primate) are dosed daily for six months. Three dose levels are chosen so that the high dose will produce significant retardation of growth or some pathological changes, the low dose is about twice the expected maximum clinical dose, while the third dose is fixed midway between the high and the low dose. The high dose level should ideally be at least 10 times the expected maximum clinical dose. During the course of the test, the following parameters are measured at regular intervals (at least every 14 days): body weight, food intake, renal function, hepatic function, haematology, pulse rate, blood pressure, and blood level of the compound tested. At the end of the test, all animals are sacrificed and autopsy performed, vital organs weighed and examined for gross changes as well as for any histological changes. Long-term treatment in man must be preceded by 3 to 12 months of chronic toxicity studies in animals.

*Special tests.* If the drug is to be used in women of childbearing age, its effect on fertility as well as its teratogenic potential must be investigated. Drugs that are to be used for chronic treatment should be tested for carcinogenicity. It has been stated that generally man is six times as sensitive as the dog, and 10 times as sensitive as the rat to the toxic effects of drugs. However, there is no pharmacological basis for such assumptions except that the smaller the animal the greater is its resting metabolism per kilogram body weight. The small animal has a big surface relative to weight from which heat is lost. To maintain body temperature relatively more heat must be produced. Hence, the metabolic rate is more in mouse (180 kcal/kg), than in rat (90), monkey (50), dog (36) and man (27).

### Acute Toxicity Test

The acute toxicity test aims at establishing the *therapeutic index*, i.e. the ratio between the pharmacologically effective dose and the lethal dose on the same strain and species (LD<sub>50</sub>/ED<sub>50</sub>). The greater the index the safer the compound and *vice versa*. Because of species variation several species of animals are used to determine the LD<sub>50</sub>. In practice, however, it is not necessary to establish LD<sub>50</sub> figures for a whole range of species. It is considered sufficiently adequate if LD<sub>50</sub> with confidence limits is established on one common laboratory species, such as mice

or rats by the standard method. The LD50 dose, thus found, is then given to guinea pigs, rabbits, cats or dogs on weight basis or better still on the basis of relative surface areas. The dose to be given to a particular species on the basis of surface area can be extrapolated by referring to Table 25.2. To determine the absolute dose for a species in the column, the absolute dose given to the species in a row is multiplied by the factor given at intersection of relevant row and column. For instance, the LD50 of crotalaburnine (CL) is 83 mg/kg i.v. in mice (Snehalata and Ghosh, 1968). Therefore, absolute dose in a 20 g mouse is 1.66 mg. Extrapolated to dog (12 kg) by surface area, the effect might be expected at a dose of (1.66 mg × 124.2) 206.17 mg as opposed to 996 mg given by ratio of body weights. The LD50 value of CL for dog is, therefore, calculated as (206.17 mg/12 kg) 17.18 mg/kg, which should be tried out in this species.

**Table 25.2**  
Surface area ratios of some common laboratory species and man\*

	20g Mouse	200g Rat	400g Guinea pig	1.5 kg Rabbit	2kg Cat	4 kg Monkey	12 kg Dog	70 kg Man
20g Mouse	1.0	7.0	12.25	27.8	29.7	64.1	124.2	387.9
200g Rat	0.14	1.0	1.74	3.9	4.2	9.2	17.8	56.0
400g Guinea pig	0.08	0.57	1.0	2.25	2.4	5.2	10.2	31.5
1.5 kg Rabbit	0.04	0.25	0.44	1.0	1.08	2.4	4.5	14.2
2kg Cat	0.03	0.23	0.41	0.92	1.0	2.2	4.1	13.0
4kg Monkey	0.016	0.11	0.19	0.42	0.45	1.0	1.9	6.1
12 kg Dog	0.008	0.06	0.10	0.22	0.24	0.52	1.0	3.1
70 kg Man	0.0026	0.018	0.031	0.07	0.076	0.16	0.32	1.0

\*From Paget and Barnes, 1964

If the LD50 is of the same order in a number of species investigated, then one may cautiously assume that a similar LD50 exists in man. If a compound is to be administered in infants less than six months of age, the LD50 in new born rats under 24 hours of age is compared with the LD50 of mature rats in order to assess any difference in sensitivity due to the age.

### Design of Acute Toxicity Test

In any screening programme, acute toxicity tests on mice are usually performed first. The compound is administered once orally or parenterally at various dose levels to groups of five to ten mice of both sexes about equal in number, which have been fasting overnight (about 18 hour). At least three or four dose levels causing less than 50 per cent, but not zero percent, and more than 50 per cent but not 100 per cent mortality should be used. The intravenous route is preferable to the intraperitoneal route, because many drugs get detoxicated by the liver when given by the latter route. Whenever possible, the solvent should be isotonic saline, and the usual volume of intravenous injection should be 1 to 10 ml/kg and maximum 50 ml/kg. Water-miscible solvents, like glycerol or propylene glycol, though may be administered intravenously, they are best avoided because of unknown influence on the test compound when given together despite parallel control studies. Solutions with widely variable pH or containing a non-aqueous solvent should be administered slowly and with care to avoid any precipitation of the material in the blood. Slow and uniform speed of injection will also avoid undue killing by a drug having predominant action on the central nervous system or on the heart.

Before the actual LD<sub>50</sub> determination, a pilot study is made on a small group of mice, mainly to select the dose range for the subsequent study. The compound is administered intravenously to pairs of mice in ascending and widely spaced doses, say, 10, 30, 100, 300 and 1000 mg/kg. The injected mice should be observed continuously for two hours, and then occasionally for further four hours and finally overnight mortality recorded. By observing the behaviour of the injected animals carefully, valuable indications of the action of the drug may be obtained, which may guide the experimenter for further testing. Convulsions during injection can usually be felt as tremor in the tail, or observed as paddling of the feet. Respiratory arrest when immediate is almost accompanied by rising of the head. Whenever such effect is observed, the injection is repeated in fresh mice taking care to inject very slowly in order to minimize such effect. A checklist for important signs is given in Table 25.1. The dose killing one out of two mice in such experiments gives a very approximate estimate of the LD<sub>50</sub>.

Another method is that, each dose is given to one animal only, and the LD<sub>50</sub> estimated from the mean of the logarithms of the smallest effective dose and the largest ineffective dose.

A simple method which is economical in animals but not in time is known as the "up and down" or "staircase" method. Two mice are injected with a particular dose, say, 50 mg/kg and observed for a period of 24 hour for any mortality. The subsequent doses are then increased by a factor 1.5 if the dose was tolerated, and decreased by a factor 0.7 if it was lethal. The maximum nonlethal and the minimum lethal doses are thus determined approximately using only about 10 mice.

Once the approximate LD<sub>50</sub>, or the range between the maximum non-lethal and minimum lethal dose is found, a final and more reliable LD<sub>50</sub> assay is planned

using at least three or four dose levels within this range with larger number of animals in each group. Following injections, the animals should be placed separately in glass cylinders or suitable containers for close observation, and should not be grouped together in the same cage, because aggregation of mice has been shown to affect the LD50 value of amphetamine and a few other drugs. The final LD50 value is determined by various procedures and expressed in terms of mg per kg. In addition, a number of information such as source of the animal, sex, age, body weight, injection time, route and solvent used, and presence or absence of any immediate reactions are also recorded for future references.

A comparison of intravenous LD50 value with the oral value gives an indication of the degree of absorption of the compound from the gastrointestinal tract. Thus, an almost identical value would indicate complete absorbability when given orally, while a higher oral LD50 value would indicate poor absorption of the compound.

*Extrapolation of the first dose to be tried in humans from the data obtained in laboratory animals.* Man is a distinct species, and it is not always true that a drug which appears safe for animals will be safe for man, or conversely, that a drug which shows alarming toxicity in animals will necessarily represent the same hazard for man. The predictability of the drug response in man might be improved not by increasing the number, but by including additional species and less pure strains of animals in the toxicity tests. On the basis of data from acute toxicity studies, the minimum effective dose producing any response is taken as the dose for calculation. If the rat was the test animal, a safe arbitrary suggestion might be two-hundredth of this dose as the total single initial dose for an average man using the same route of administration. If the dog was the test animal, then one-tenth of this dose might be used. A wide safety margin, however, can be obtained by transferring toxicity in terms of surface area, and activity in terms of body weight.

Table 25.3 presents commonly used terms for toxicities along with the dose equivalents both in rats and in humans.

**Table 25.3**  
**Commonly used terms for toxicities**

<i>Commonly used terms</i>	<i>LD50 (Single oral dose per kg in rats)</i>	<i>Probable lethal dose for man</i>
Extremely toxic	1 mg or less	65 mg
Highly toxic	1-50 mg	4 g
Moderately toxic	50-500 mg	30 g
Slightly toxic	0.5-5g	250 g
Practically nontoxic	5-15 g	1 kg
Relatively harmless	15 g or more	> 1 kg

**CALCULATION OF LD50 VALUE**

*Graphical method:* This method is simple and accurate enough in most of the cases and should always be tried first. The observed percentage mortality is converted into probit by referring to the Table 25.4 and the values thus obtained



are plotted against log dose. The LD50 value and its standard error may be determined from the graph if the line is straight enough.

Table 25.4  
Transformation of percentages to probits<sup>†</sup>

%	0	1	2	3	4	5	6	7	8	9
0	-	2.67	2.95	3.12	3.25	3.36	3.45	3.52	3.59	3.66
10	3.72	3.77	3.82	3.87	3.92	3.96	4.01	4.05	4.08	4.12
20	4.16	4.19	4.23	4.26	4.29	4.33	4.36	4.39	4.42	4.45
30	4.48	4.50	4.53	4.56	4.59	4.61	4.64	4.67	4.69	4.72
40	4.75	4.77	4.80	4.82	4.85	4.87	4.90	4.92	4.95	4.97
50	5.00	5.03	5.05	5.08	5.10	5.13	5.15	5.18	5.20	5.23
60	5.25	5.28	5.31	5.33	5.36	5.39	5.41	5.44	5.47	5.50
70	5.52	5.55	5.58	5.61	5.64	5.67	5.71	5.74	5.77	5.81
80	5.84	5.88	5.92	5.95	5.99	6.04	6.08	6.13	6.18	6.23
90	6.28	6.34	6.41	6.48	6.55	6.64	6.75	6.88	7.05	7.33

<sup>†</sup> Abridged from Table IX of Fisher and Yates: *Statistical Tables for Biological, Agricultural and Medical Research*, published by Oliver and Boyd, Edinburgh, and by permission of the authors and publishers.

*Example:* Determination of intravenous LD50 value of crotalaburnine (CL) in mice (Snehalata and Ghosh, 1968) by the graphic method of Miller and Tainter (1944). The results are presented in Table 25.5. Before plotting, the percentage dead for 0 and 100 are corrected. The probit values are plotted against log doses, and then the dose corresponding to probit 5 (50%) is found out (Fig. 25.1). In the present case, the value was found to be 79.5 mg/kg.

The approximate standard error of LD50 value is obtained by the formula:

$$\text{Approx. S.E. of LD50} = (\text{Log LD84} - \text{Log LD16}) / \sqrt{2N}$$

where N is the total number of animals employed in the two groups. The log values are obtained from the line on the graph corresponding to probits 6 and 4.

Table 25.5  
Results of acute intravenous toxicity of CL in mice\*

Group	Dose mg/kg	Log dose	Dead/Total	Dead %	Corrected %**	Probit
1	64	1.81	0/10	0	2.5	3.04
2	71	1.85	2/10	20	20	4.16
3	81	1.91	4/10	40	40	4.75
4	90	1.95	9/10	90	90	6.28
5	100	2.00	10/10	100	97.5	6.96

\* Snehalata and Ghosh, 1968

\*\* Corrected formula : for the 0% dead :  $100(0.25/n)$ ; for the 100% dead :  $100[(n-0.25)/n]$ , where n is the number of animals in the group.

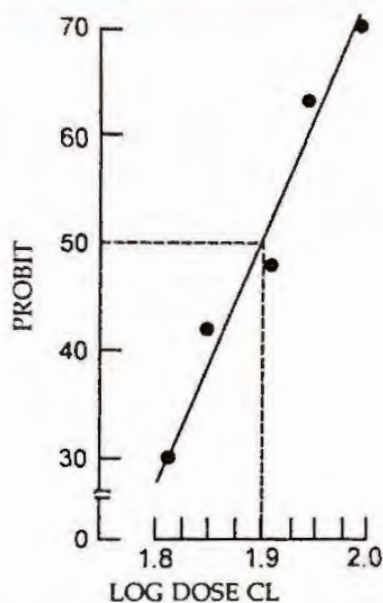


Fig. 25.1. Graphical method of determination of LD50. The results have been plotted from the Table 25.5. The LD50 value was found to be 79.5 mg/kg.

*Arithmetical method.* The LD50 may be calculated by Karber's method that does not involve any plotting of dose-response curve. It is the simplest and rapid though crude method of deriving LD50 value particularly when the number of animal is small. The interval mean of the number dead in each group of animals is used as well as the difference between the doses for the same interval. The product of the interval mean and the dose difference is obtained. Results from doses larger than the least dose lethal to all in a group and from doses smaller than the maximal tolerated dose are excluded. The sum of the product is divided by the number of animals in a group and the resulting quotient is subtracted from the least lethal dose in order to obtain the LD50 value. The results of the same CL data have been presented in Table 25.6. The LD50 value of CL (81 mg/kg) obtained by this method compares favorably with the value (79.5 mg/kg) obtained by the graphical method.

Table 25.6  
LD50 determination by Karber's method

Group	Dose mg/kg	No. of animals	Dose difference (a)	Dead	Mean mortality (b)	Product (a × b)
1	64	10	—	0	—	—
2	71	10	7	2	1	7
3	81	10	10	4	3	30
4	90	10	9	9	6.5	58.5
5	100	10	10	10	9.5	95
						190.5

$$LD50 = 100 - (190.5/10) = 81 \text{ mg/kg (approx)}$$

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# Some Standard Physicochemical Techniques

## PHOTOMETRY

### General Principles

When white light is allowed to pass through a solution which absorbs radiation of a particular wavelength, the transmitted light is complementary to that of the absorbed light (complementary colours: violet and yellow, blue and orange, green and red). The amount of light absorbed is expressed as absorbance  $A$  (also known as extinction  $E$ ).

$A = \log(I_0/I) = kcl$ , where  $I_0$  is the intensity of incident light,  $I$  is the intensity of transmitted light,  $k$  is a constant for the substance,  $c$  concentration of the substance and  $l$  length of light path.

$$k = A/cl.$$

When  $c$  is expressed in moles/litre and  $l$  in cm, then  $k$  is known as molar absorptivity or molar extinction coefficient ( $\epsilon$ ). Values of  $\epsilon$  are useful to characterize compounds and to establish their purity.

## COLORIMETRY

In this, absorption of light is measured only in the visible range (300 to 750 m $\mu$ ) by using a tungsten lamp as the light source. Light from this is allowed to pass through a colour filter (the colour of which is complementary to that of the absorbing substance) in order to select the desired range of wavelength. It is then passed through a slit, and then through a tube containing the substance in solution. The intensity of the emerging light is measured using a photo-electric device attached to a galvanometer which is calibrated in extinction values.

Initially the sample tube is filled with the solvent, and by adjusting the slit width, the galvanometer reading is brought to zero. Then the absorbance of standard as well as the test solution is measured; from this the concentration of the test solution can be calculated. A better method is to plot a calibration curve (absorbance vs concentration) of the standard by using different concentrations of the standard solution. By measuring the absorbance of the test solution, the concentration can be found out using the graph.

### SPECTROPHOTOMETRY

In spectrophotometer, the measurement can be made at any point in the electromagnetic spectrum (UV, Visible or IR). However, for practical purposes 200 to 750  $m\mu$  range is used. Unlike colorimeter, spectrophotometer has the advantage that even the compounds which are colourless but which absorb radiation selectively in the UV region can be measured.

The instrument consists of as a light source, hydrogen lamp for UV radiation (200 - 340  $m\mu$ ) and tungsten lamp for visible range (300 - 750  $m\mu$ ), and a monochromator (prism or diffraction grating) instead of a colour filter, which selects radiation of a desired wavelength. The other parts are similar to that of the colorimeter.

For qualitative study, the absorbance of a compound at different wavelengths can be measured, and by plotting a graph of  $A$ ,  $\epsilon$  or  $\log \epsilon$  vs. wavelength, the absorption spectrum is obtained. The absorption spectrum of a compound is characteristic of the compound. Hence, it is useful in the identification and determination of purity and structure of a compound.

The wavelength at which maximum absorption occurs is known as  $\lambda$  max. As in colorimetry, a calibration graph of absorbance (at  $\lambda$  max) vs. concentration of the standard is useful for the quantitative measurement.

### FLUORIMETRY

Some molecules when excited by the absorption of radiation soon return to the ground state by emitting fluorescence at longer wavelength (lower energy). Fluorescence usually consists of a number of different wavelengths, and constitutes a fluorescence spectrum that is characteristic of a compound. The intensity of fluorescence is proportional to the concentration of the fluorescing substance. Hence, by observing the intensity of fluorescence, the concentration of the substance can be found out using a suitable standard.

Fluorimetry has a greater overall specificity than spectrophotometry for each organic molecule. This is because only fewer molecules exhibit fluorescence. Moreover, in fluorimetry both the wavelength of the absorbed and emitted radiations are taken into consideration, which are characteristics of the particular compound.

### FLAME PHOTOMETRY

Certain metals when excited by the thermal energy emit light with wavelengths characteristic of the metals. The intensity of emitted light can be measured using a flame photometer.

The temperature at which the metals get excited varies from metal to metal. Thus Li, Na, K and Ca require temperature of 1700°C for excitation, whereas Cu, Ag and Fe require still higher temperature.

The flame photometer has a non-luminous flame as the heat source. The test solution containing the metal ions is sprayed into the flame with the help of an atomizer. The intensity of the emitted light is measured by a photoelectric device. Finally, the concentration of the test solution is found out using a calibration curve of the standard as before.

### CHROMATOGRAPHY

The chromatographic technique is used for the separation, purification and identification of compounds present in a mixture. The principle is based on the differential distribution of a mixture between two phases, the fixed or the stationary phase and the moving or the mobile phase. The stationary phase is an adsorbent like activated alumina, silica gel, cellulose and ion-exchange resins (adsorption chromatography), or it may be a solvent that dissolves the solute (partition chromatography). The mobile phase is a liquid or a gas.

### COLUMN CHROMATOGRAPHY

The column chromatography is used for the separation of large quantity of samples.

*Adsorption chromatography.* In this, the adsorbent is packed in a glass tube having glass wool or cotton at the bottom. The mixture of drugs present in a solvent is poured on the top of this column. Then more of the solvent is slowly added. The drug particles move down at different rates depending on their affinity for the adsorbent.

If the drugs are coloured, then different layers containing these drugs can be easily identified. The column can be extruded and each layer separated. If the compounds are colourless, suitable methods (spraying reagents which develop colours, inspection under UV light, etc.) can be employed to locate the layers. Finally, each substance can be extracted using a suitable solvent.

Instead of extruding the column, the substances can also be separated by pouring more of the solvent at the top of the column so that the dissolved substances come out at the bottom one after another.

*Partition chromatography.* It is similar to the adsorption chromatography, but the only difference is that both the mobile and the stationary phases are liquids (immiscible). The stationary phase consists of a liquid adsorbed on the column so as to give a larger surface area, which in turn gives a better separation.

### PAPER CHROMATOGRAPHY

It is used for separation and identification of small quantity of a sample. In paper chromatography, the adsorbent is a strip or sheet of special paper that serves as the stationary phase. The mobile phase (liquid) is allowed to move upward (ascending chromatography) or downward (descending chromatography). The paper is suspended in a vapour-tight chamber. One end of the paper is in contact

with the mobile phase. The mixture to be separated is applied in minute quantity over a spot on a line drawn a few centimeters away from the paper-end which is in contact with the mobile phase. The chamber is sealed to allow equilibration (saturation) of the chamber and the paper with the solvent vapour. After the solvent front has moved over a desired distance, the location of the solvent front is marked, and the paper is allowed to dry.

The area of each separated compound can be identified as in the case of column chromatography. The  $R_f$  value is calculated as follows:

$R_f = dc/ds$ , where  $dc$  = distance travelled by the compound, and  $ds$  = distance travelled by the solvent front. The  $R_f$  value is useful in the identification of the compound.

### THIN LAYER CHROMATOGRAPHY

It is used for separation and identification of very small quantity of samples. In this, the adsorbent (stationary phase) is applied over a glass plate as a thin layer. The drug spots are made near one end, which is dipped in the solvent (mobile phase). The plate is kept in a vapour-tight chamber and the  $R_f$  values of the compounds calculated. The spots may also be removed for quantitative analysis.

### GAS CHROMATOGRAPHY

Gas chromatography requires sophisticated apparatus, and is used for both qualitative and quantitative analyses.

In gas chromatography, the mobile phase is an inert gas like helium. The stationary phase is either a liquid coated on to an inert material (Gas-Liquid Chromatography) or a solid adsorbent (Gas-Solid Chromatography).

The mixture to be separated is vaporized and is carried by the mobile phase into the chamber containing the stationary phase. If it is difficult to vaporize the compounds, then their derivatives (acetylated, methylated, etc.) that vaporize easily can be prepared. The chamber is also kept at a high temperature (150-350°C) so that the vapor does not condense. The compounds in the mixture get separated and they come out of the chamber one by one. A detector present at the outlet emits a signal proportional to the concentration of the outgoing substance. The detector is coupled to an automatic recording device, which plots a graph showing the intensity of signal against time. Using this graph as well as a standard calibration curve, the nature and the concentration of the compounds can be determined. The compounds coming out of the chamber can also be collected in a cold trap for further analysis.

The advantages of gas chromatography over the other methods are that the separation is achieved more rapidly, and that there are various sensitive 'on-line' methods of detection and quantitation of drugs readily available.

### HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

High performance or high pressure liquid chromatography (HPLC) is a form of liquid chromatography in which the solvent (mobile liquid phase), instead of being allowed to drip through the column under gravity, is forced through under high pressure so as to run it much faster. HPLC is a powerful tool used in the separation, identification, and quantitation of the compounds present in a sample dissolved in the solvent.

HPLC instrument consists of a reservoir for the solvent (most commonly methanol and acetonitrile), a pump for producing high pressure, an injector for introducing the sample, a separation column packed with sorbent (small spherical silica particles), and a detector (such as UV, fluorescence, electrochemical, etc).

The compounds are separated by injecting a sample mixture onto the column; the different fractions in the mixture pass through the column at different rates due to the differences in their partition behaviour between the mobile liquid phase and the stationary phase.

*Normal-phase* partition chromatography uses a polar stationary phase, such as a bonded siloxane with a polar functional group, and a nonpolar organic solvent, such as n-hexane, methylene chloride, or chloroform as the mobile phase.

*Reverse-phase* partition chromatography uses a relatively nonpolar stationary phase and a polar mobile phase, such as methanol, acetonitrile, water, or mixtures of these solvents. Reverse-phase chromatography is the most common form of liquid chromatography where wide ranges of analytes dissolve in the mobile phase.

A recorder is used to display the results, and an integrator for performing the calculations. The elution of the fraction from the column is detected as a peak in the chromatogram. The retention time at the peak is used in the identification of the fraction, while the area under the peak determines the amount of the fraction in the sample.

The advantages of HPLC over other forms of liquid chromatography are: i) compounds in trace concentrations as low as parts per trillion (ppt) can be detected, ii) analysis done within much shorter time, iii) achieves a higher degree of resolution, that is, more complete separation of the fractions, iv) stationary columns can be re-used without waiting for their regeneration, v) results are highly reproducible, and vi) permits both instrumentation and quantitation to be automated.

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# Immunoassays

## RADIOIMMUNOASSAY (RIA)

Sensitive, precise and specific methods for measuring the concentration of substances and drugs in blood are needed for analysis of their distribution and metabolism, and for monitoring their blood levels. Classical bioassay methods have been found to be too insensitive to permit accurate measurements of nanogram and picogram quantities to be met under clinical conditions. This was, however, overcome by the advent of radioimmunoassay (RIA), which was first developed for insulin by Yalow and Berson (1960).

The concept of RIA is based on the principles of law of mass action and isotopic dilution of a characteristic antigen-antibody reaction. This occurs when the equilibrium state is allowed to set in the reaction between the antigen (component to be measured), restrictive amounts of its specific antibody, and a corresponding antigen-antibody complex in a system that includes trace amount of labelled antigen. At this state, as the amount of unlabelled antigen is increased, there is a competitive inhibition of labelled antigen bound to the antibody, provided the same amount of labelled antigen and antibody are used throughout the assay procedure. As a result of this competitive inhibition, the ratio of labelled bound antigen (B) to the free (F) antigen denoted as B/F is diminished as the concentration of the unlabelled antigen is increased.

Since the final expression of results is based on the counts of labelled fraction bound to the antibody, efficient methods for effective separation of bound and free antigen fractions should be obtained by various methods such as electrophoresis, chromatography, adsorption on to the dextran coated charcoal, or by the use of double antibody procedure. The concentration of the unknown substance is determined from a standard curve in which known quantities of the standard antigen are added to the system.

As the substance to be assayed should possess antigenicity, it was initially useful only in establishing RIA procedures for protein and peptide hormones, Australia antigen, etc. However, in subsequent years, methods were developed by covalently linking several lower molecular weight substances, such as digitoxin, morphine, pethidine, chlorpromazine, steroid hormones, etc. to bovine serum albumin and other proteins. The antibody directed against these coupled antigens maintained their immunological specificity of agglutination reaction with the low molecular weight drugs and hormones otherwise termed *haptens*. Using the above techniques, almost all the drugs currently in therapeutic use including most of the hormones could be put into RIA system for estimation and monitoring their blood levels.

Though RIA method is accurate and highly sensitive, it is quite cumbersome, is costly and cannot be employed in smaller centres due to heavy cost of labelled materials, and in the maintenance of antibodies and counting instruments, such as liquid scintillation  $\beta$  counters and gamma ray spectrometers. Moreover, RIA cannot discriminate between biologically active and inactive substances, as the reaction is purely dependent on immunogenic property of antigen-antibody coupling. If this immunogenic property continues to be retained in a biologically degraded and inactive component or metabolite, RIA system employed for such a compound may give false positive results. Nor will it be of much use, if a drug becomes biologically active *in vivo* only. Under these circumstances, biological assay should be the appropriate technique.

### ENZYME IMMUNOASSAY (EIA)

This procedure was developed as an alternative to overcome the drawbacks encountered in the RIA system. It offers a method for a sensitive and reproducible detection of antigen-antibody union with relatively inexpensive reagents, and without any risk of radiation.

Instead of a radioactive label, EIA makes use of an enzyme as a marker, the activity of which can be assayed using colorimeter or spectrophotometer. The enzyme is first coupled either with the antigen or the antibody depending on which is being measured. The enzyme labelled substance and that present in the fluid to be assayed compete for the binding sites. Following the union between antigen and antibody, the enzymatic activity of the label is assayed by the addition of a suitable substrate. The enzymatic activity present in the system is directly proportional to the amount of enzyme label present either on the antigen or on the antibody, and inversely proportional to the amount present in the fluid. A major modification of EIA has been the immobilization of either antigen or antibody on polystyrene or polyvinyl surfaces, thus generating an immunoadsorbent, the procedure being called *enzyme linked immunosorbent assay* or ELISA. Immobilized standard antigen has been used to quantitate antibiotics by the use of labelled antiglobulins. The enzymes generally employed as markers are horse radish peroxidase, penicillinase, malate dehydrogenase, or glucose 6-phosphate dehydrogenase, the activities of which can be assayed photometrically.

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## Plant Extraction Methods\*

Plants continue to remain an important source of medicines for human ailments. The use of plant products in the treatment of various diseases in humans and animals can be traced to various old civilizations in different parts of the world. This practice has evolved as traditional system of medicine in many countries. Subsequent scientific validation of traditional medicines has contributed significantly to the development of modern medicine. Modern analytical techniques have enabled us to isolate and identify active ingredients from plants. Many active principles such as atropine, morphine, reserpine, etc. derived from plants have been employed in modern medicine. In addition, these compounds served as lead molecules for the development of many more useful drugs. Thus, the investigation of plants for active ingredients has always fascinated the chemists and pharmacologists alike. The avid interest in plant chemistry (phytochemistry) has rewarded us with more than 10,000 plant alkaloids (Harborne 1998), a few thousand flavonoids and numerous other types of active constituents. Hence, the investigation of medicinal plants is being actively pursued all over the world. An overall idea regarding the criteria for selection of a plant for investigation, processing the parts of the plant, methods of extraction etc. will be useful to the new investigator venturing into this area of research.

### Selection of Plant Material

*Traditional claim.* By convention, the main criterion for selection of a plant for investigation is always a mention of its use in traditional systems of medicine or folklore practices. Such a selection provides justification for the scientific pursuit as well as for validation of the claims made in traditional systems. Sometimes the plant selection is made based on the presence of some specific compound(s). Apart from this, random selection of a plant for analysis is being adopted in some laboratories.

*Availability.* It is preferable for a new investigator to choose a plant available locally. This will obviate the need for transport of plant material from far away places leading to its deterioration or spoiling during transit. However, the mere presence of a plant material in abundance should not be the sole criterion for starting an investigation. Other related species of the traditionally used plant may also yield similar active constituents and exert similar properties.

\* Contributed by S. Viswanathan, E. Sukumar, and S. Ramaswamy

*Authentication of plant material.* The correct botanical identity of the plant selected for investigation should be established beyond doubt. It is preferable to seek the help of a field botanist during the collection of a plant material, which should be authenticated by an expert taxonomist. Voucher specimens should be preserved in the institution as well as deposited in a recognized herbarium for future reference.

*Plant parts to be used.* In general, the same part of the plant that has been used in traditional medicine should be chosen for investigation. Leaves, flowers, fruits, stem, bark, seeds and roots of a plant are used in many instances to treat diseases in folklore practice. The material collected should be free from any extraneous impurities, as well as from parasitic or fungal contamination that may otherwise yield misleading result.

*Processing of plant material.* Ideally, fresh plants should be used for extraction. However, when this is not possible, the plant material should be dried in shade at room temperature away from the direct sunlight. Air drying considerably reduces the water content in the plant material, which otherwise is a hindrance to the extraction, preservation and isolation processes. The dried plant part may be stored in airtight containers for a reasonable period till extraction.

### Extraction

Extraction refers to the separation of medicinally active ingredients from plant material employing selective solvents and standard procedures.

*Secondary metabolites.* Plants in addition to the production of primary metabolites also synthesize a variety of secondary metabolites. These secondary metabolites like anthocyanin pigments, flavonoids, alkaloids, terpenes and glycosides etc. are useful in pollination, protection of plants from infection and from grazing animals besides playing significant role in plant metabolism. Interestingly, these secondary metabolites are endowed with many therapeutic benefits applicable to humans. Hence, the extraction procedure is primarily targeted towards the separation of secondary metabolites from plants.

*Extraction procedures.* The use of a known herb in powder form or as decoction is a legendary practice in traditional medicine. Modern science has suitably modified this technique to extract different fractions with various solvents, and isolate active compounds by adopting suitable methods. A variety of solvents are used to extract different types of active ingredients from the plants. These solvents penetrate the plant cells to dissolve the chemicals bringing them in solution. The dried plant material is coarsely powdered before extraction so that there is good penetration of the solvent into the plant tissues. The following methods are usually employed in the laboratories for extraction of active constituents from plants.

### Cold Extraction

*Maceration.* Maceration is a method of cold extraction of a plant material with

a solvent. Shade dried and coarsely powdered plant material is charged in a stoppered glass container, and filled with a suitable solvent till the plant material is completely soaked. Frequent shaking / agitation will help in the uniform spread of the solvent. The container is kept at room temperature for 3-4 days. The extract is decanted and filtered. More solvent is poured into the container and the process repeated for complete extraction. The filtered extracts are combined and the solvent is removed by distillation over boiling water bath, and concentrated at low temperature using a rotary flash evaporator under vacuum. The condensed extract as such may be used for pharmacological screening, or alternatively it may be further processed to separate the active chemical constituents. Maceration is the most commonly employed method of extraction being very useful in the extraction of thermo-labile compounds. However, a large volume of solvent is needed for repeated extraction, and some extract is retained in the plant material. To avoid this, the soaked plant material is taken out and squeezed to express all the extract.

*Percolation.* This is another widely used method for plant extraction. The equipment used is a conical glass / porcelain vessel with a tap at the bottom (percolator). The plant material along with solvent is filled in the vessel and closed. After standing for 4 – 24 h, the tap of the vessel is opened to drain the extract. Additional solvent is added to the vessel and the process is repeated. The marc is then pressed and the expressed solution is added to the extract which can be condensed as described above. In the percolation method, hot solvent may be employed, and the extract is continuously collected at the bottom. This approach may reduce the time required for complete extraction. Percolation is more efficient method of extraction than maceration since it is a process in which the saturated solvent is continuously replaced by fresh solvent. However, finely ground plant material may result in clogging of the percolator tap, which may be avoided by placing a piece of cotton wool at the bottom of the percolator prior to loading of the plant material.

### Soxhlet Extraction

This is a process of continuous extraction in which hot solvent percolates through the powdered plant material and gets recycled. Soxhlet assembly (Fig. 28.1) is made of three detachable parts—a round bottomed flask that holds the solvent, a body of extractor with a side tube, and a siphon facility. The mouth of the extractor is connected to a reflux condenser for water circulation. The coarsely powdered plant material is packed in the extractor either on a cotton bed, or in a thimble, or packed in filter paper. Initially, the extractor containing the plant material is filled with solvent at room temperature, and allowed to siphon once before heating. The round bottomed flask is placed over a water bath, and a few freshly activated porcelain bits are slipped in to the flask to avoid bumping. The solvent is heated to boiling; the vapour passes through the side

arm up into the reflux condenser, gets condensed and drips into the plant material. Once the extraction chamber is filled with the extract, the siphoning system pushes entire contents into the round bottomed flask. The cycle of solvent evaporation and siphoning back continues a number of times resulting in efficient extraction. The extract collects into the lower flask and becomes more concentrated with repeated cycles. The vapour rising from the heated extract is pure solvent vapour, which condenses on the material again. The advantage of this system is that a limited quantity of solvent is recycled to get the plant extract containing the chemical principles. However, repeated heating of the extract may affect thermo-labile compounds.

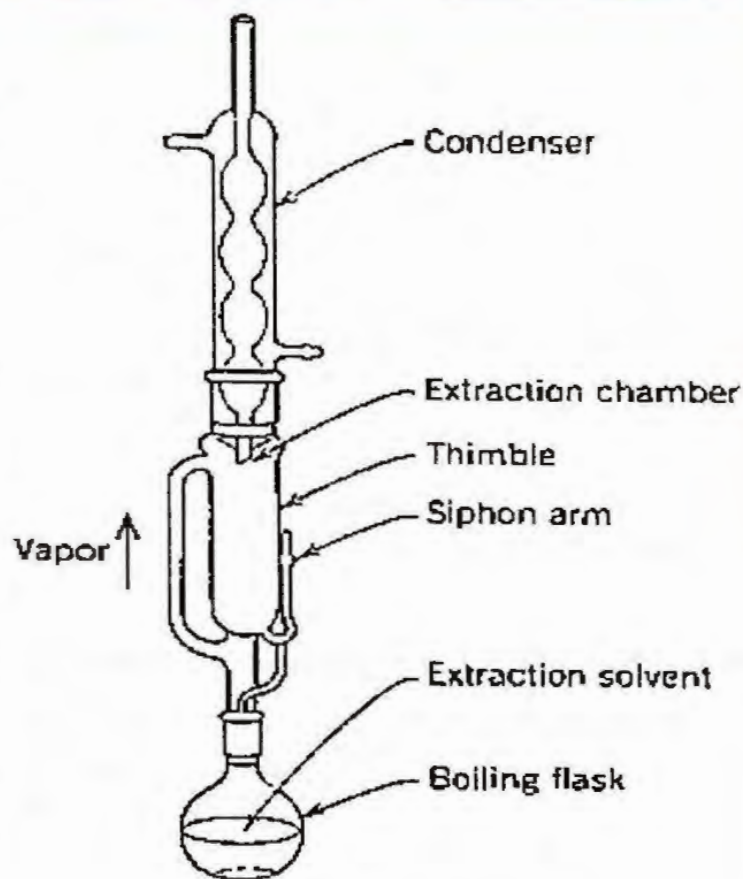


Fig. 28.1 Soxhlet apparatus

*Sequential exhaustive extraction / serial exhaustive extraction.* This procedure is very useful in bioassay-guided fractionation of active principles. In this method, the plant material is extracted successively using a soxhlet apparatus with solvents of increasing polarity from a low polar solvent like *n*-hexane or petroleum ether to a high polar solvent like methanol. Thus, it is possible to fractionate different types of compounds with each solvent according to their polarity. The extract obtained from each solvent may be concentrated and used for pharmacological screening and for chemical tests. Further steps in the isolation and identification of the active principles are made easy by this technique, since a preliminary stage of separation is already achieved. While changing the solvent, it is necessary to dry the plant material before placing it into the soxhlet.

*Decoction.* This is a conventional method adopted in all traditional systems of medicine. Fresh or dry plant material is soaked in water and boiled for about 15 minutes; the extract is then cooled and filtered. In traditional systems, the decoction is used as such or diluted further and used as a medicament. The water soluble and thermo-stable constituents of medicinal plants are extracted by decoction,

which may be further concentrated and subjected to chemical testing and pharmacological screening.

*Concentration of the extract and its preservation.* For further usage, the large volume of extract obtained by the above procedures is condensed by removing the solvent by distillation. Alternatively, a rotary flash evaporator can also be used to concentrate the extract. This apparatus employs low pressure and low temperature thus ensuring complete removal of solvent from the extract without affecting thermo-labile compounds. Lyophilisation is another method employed for further concentrating the extract. By using a lyophiliser, the extract may be obtained in a powder form that can be preserved and reconstituted later with suitable solvent for pharmacological screening. In general, the concentrated extract is like a paste in consistency. This should be preserved in air tight container and stored in a refrigerator. If the extract is hygroscopic, it may be stored in a vacuum desiccator.

### Solvents used for Extraction of Active Principles

An important guideline for choosing a suitable solvent for extraction of an active constituent often follows the principle "like extracts like" (Bart, 2011). Low polar solvents like *n*-hexane or petroleum ether mainly extract compounds like wax and fats, while high polar solvents like methanol extracts compounds of high polarity such as glycosides and saponins (Fig. 28.2). Other additional factors that determine the selection of solvent include recoverability of solvent, toxicity, flammability, thermal and chemical stability. In other words, the solvent should be non-toxic, not easily flammable and easily removable by distillation.

*Hexane and petroleum ether.* These are least polar solvents useful in extracting chlorophyll, fat and plant waxes. They are mainly employed to remove the above constituents from plants which may otherwise hinder the separation of bio-active components like alkaloids, terpenoids, steroids and flavonoids etc.

*Benzene and di-ethyl ether.* These solvents are of low polarity and useful in extracting terpenoids, steroids and coumarins.

*Chloroform and acetone.* These are slightly more polar compared to the above solvents, useful in the extraction of terpenoids, steroids and alkaloids.

*Ethyl acetate.* The next higher polar solvent ethyl acetate is useful in extracting phenolic compounds including flavonoids and glycosides.

*Alcohol.* Ethyl and methyl alcohols are extensively used for extraction of active constituents from plants. Both the solvents are polar in nature, and extract major secondary metabolites from the plants. The extract obtained using methyl or ethyl alcohol is quite stable and can be stored without microbial contamination unlike aqueous extract. In most laboratories, the pharmacological activity of a plant material is determined initially using the alcoholic extract. If any useful activity is detected, then the extract is subjected to further bioassay-guided fractionation. Saponins, lactones, glycosides and poly-phenolic compounds like anthocyanins,

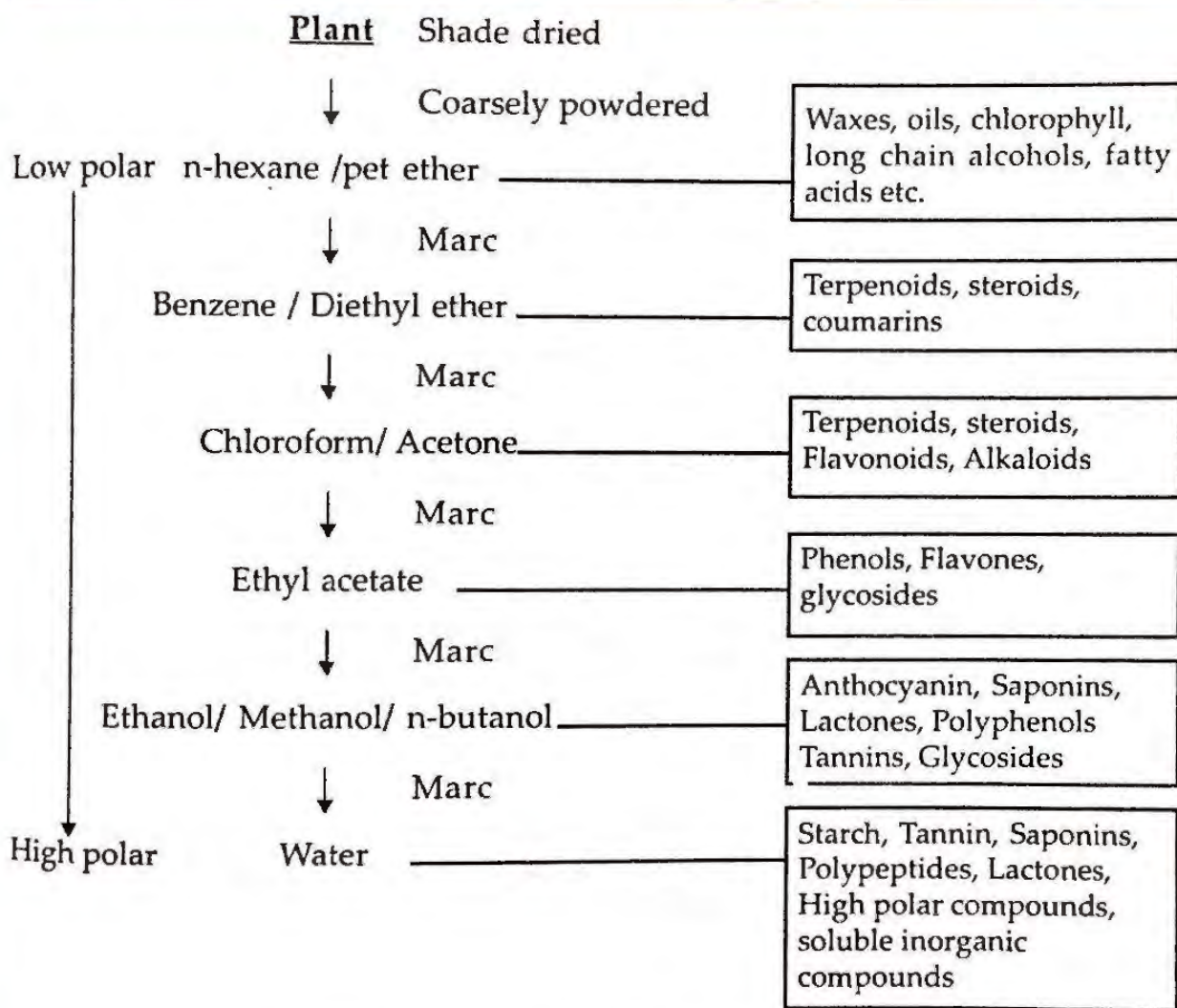


Fig. 28.2 Solvents used for extraction of active principles

flavonoids and tannins are some of the major phytochemicals extracted by alcohols. In some instances, water is added to the alcohol up to 50% to increase the polarity. The extract thus obtained is called a hydro-alcoholic extract.

**Water.** Water is widely used in various traditional systems of medicine to prepare decoction that contains most of the active constituents from plants. Water preferentially extracts high polar compounds like tannins, plant pigments, etc. that may require some special post-treatment for further purification. Generally, the solvent is evaporated to get a small volume of condensed extract, which is suitable for further chemical and pharmacological screening. However, water requiring high heat for evaporation is very difficult to remove completely compared to the organic solvents. Further, aqueous extracts being hygroscopic are prone to bacterial and fungal contamination. Finally, water soluble impurities such as inorganic salts may pose challenges in further purification by chromatography or crystallization (Bart, 2011). Thus, water employed alone is not a preferred solvent for extraction of the secondary metabolites or active constituents from medicinal plants.



*Purification of solvents.* All the organic solvents need to be pure as far as possible in order to achieve effective extraction besides unwanted interference from other compounds. For purifying solvents, standard method such as distillation is used.

### Methods of Extraction

In general, two methods are employed in the isolation of active principles from plants. In one method, the plant material is extracted with a high polar solvent like alcohol. The extract thus obtained is called 'total extract' since most of the chemical constituents / secondary metabolites from low to high polarity are eluted by alcohol. In the other method, wax, chlorophyll and fat are first removed from the plant material by using *n*-hexane or petroleum ether. Then the plant material is successively extracted with chloroform, acetone, ethyl acetate and alcohol to isolate the desired constituents. The extracts thus obtained using different solvents or the total alcoholic extract are further subjected to pharmacological and chemical screening (Fig. 28.3). Chromatography techniques are useful in the separation of individual chemicals from the extract. Pure compound thus separated from the extract is further analysed for its chemical nature and structure by employing various spectroscopic procedures.

### Chemical Tests

The pure compound isolated from the plant extract is subjected to different chemical tests using various reagents to identify and characterise its nature *viz.* alkaloid, terpenoid, steroid, flavonoid etc.

### Elemental Analysis

A small amount of the pure isolated compound is used for this analysis, which is also known as CHN analysis. The percentage of carbon, hydrogen and nitrogen as well as the chemical formula of the compound is determined.

### Infra-Red (IR) Spectroscopy

This technique is useful in identifying the presence of functional groups (such as hydroxyl, carbonyl, C-O-C etc.) as well as un-saturation in a compound. The molecules in the sample absorb radiations of specific frequencies that are characteristic of their structures. A small quantity of the compound (2–5 mg) is sufficient for the study. The substance is dissolved in a suitable solvent like *nujol* or made a pellet with KBr, and the IR radiation is passed through the substance. The emerging radiation is recorded in a chart. From the nature of the absorption bands, the functional groups, un-saturation etc. are identified.

### Ultra-Violet (UV) Spectroscopy

Ultra-violet spectroscopy deals with the study of absorption of UV radiation by compounds in the region 200 – 400 nm. Coloured compounds absorb radiation

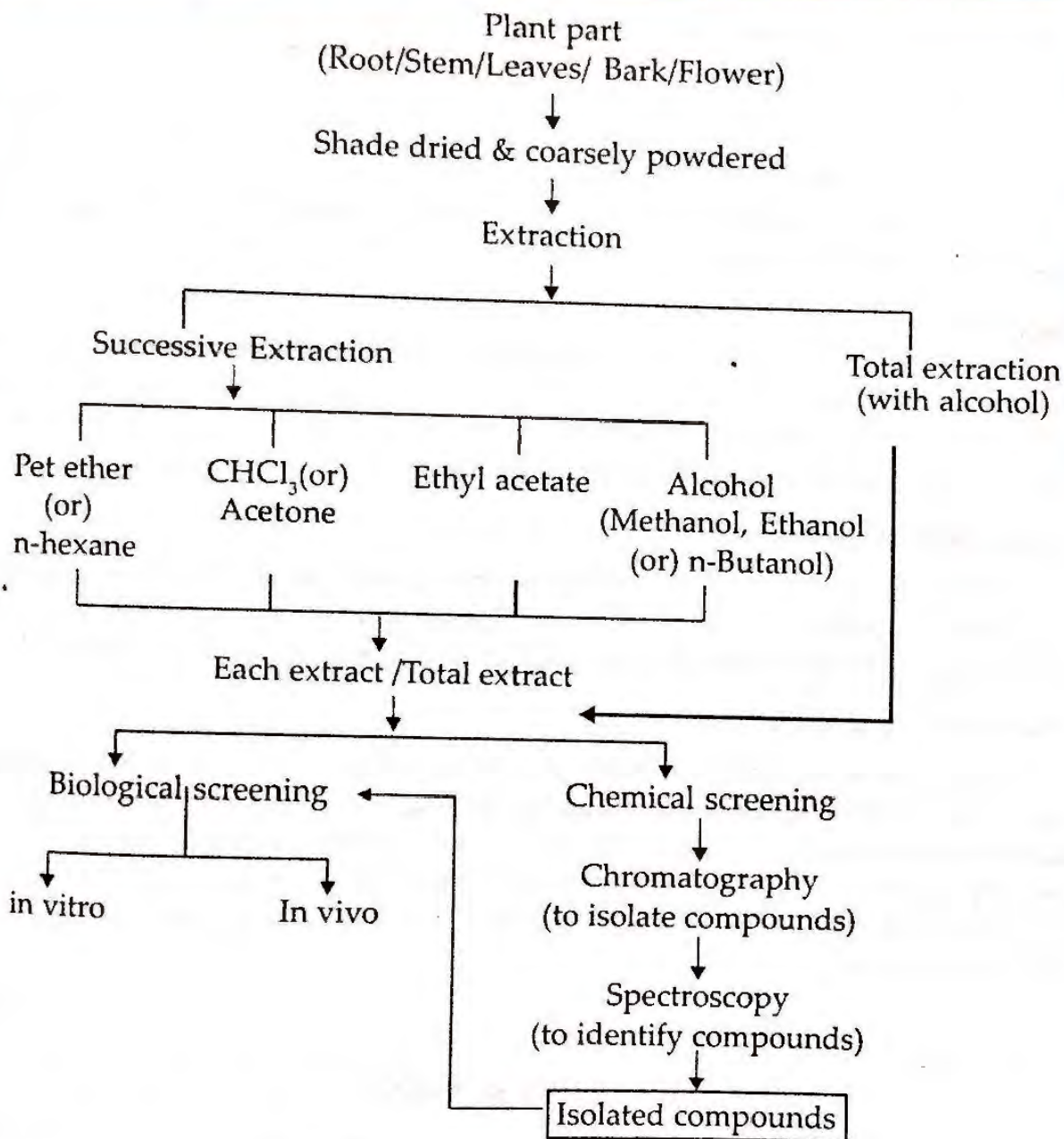


Fig. 28.3 Methods of plant extraction

in 400 – 800 nm region, while colourless samples absorb radiation in UV region. This technique is used to get information on conjugation in organic compounds, the presence or absence of un-saturation, as well as the presence of hetero atoms such as sulphur, oxygen, nitrogen or halogens.

### Nuclear Magnetic Resonance (NMR) Spectroscopy

*Proton magnetic resonance (PMR or <sup>1</sup>HNMR)*. The nature of hydrogen atoms in a compound is identified by this method. Depending upon other hydrogen atoms present in the surrounding carbon atoms, each hydrogen atom splits in the

magnetic environment giving singlet, doublet, triplet or multiplet in the chart. By studying the splitting patterns, the nature of hydrogen atoms are identified.

*Carbon magnetic resonance (CMR or  $^{13}\text{C}$ NMR).* The nature of carbon atoms (primary, secondary, tertiary or quaternary) is inferred by this method. The small amount of  $^{13}\text{C}$  isotope of the carbon atom present along with the  $^{12}\text{C}$  isotope, responds in the magnetic environment giving characteristic splitting pattern depending upon the nature of carbons present in the molecule.

### Mass Spectroscopy

In this analytical technique, the molecules present in the compound are ionized to generate molecular fragments that are measured for mass:charge ratio, and is revealed as the peak in the spectrum. By knowing the molecular ion peak, other peaks are identified as also the elemental composition of the molecule.

### X-Ray Crystallography

With pure crystalline compounds, this gives information on the three dimensional arrangement of atoms in a molecule. By using computer aided programmes, the bond lengths and angles are also arrived at by this method.

### Structure Elucidation

The information obtained employing all the methods described above enables one to arrive at the structure of the compound isolated from the plant extract. The advantage of structure elucidation is to obtain the compound in larger amounts by employing synthetic procedures. Structural modifications of the isolated compound by desired substitution or by preparing new derivatives may yield a new array of pharmacologically active molecules.

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# Alternatives to Animal Experiments

The scientific and legislative authorities and animal rights activists throughout the world have been demanding the abolition of the animal experiments in the laboratory advocating the development of some alternatives. According to Philippe Busquin, European Research Commissioner, the use of animals to test drugs is unfortunately necessary to safeguard human health. Striking a balance between the two views, Russell and Burch in 1959 developed the concept of 3 R's alternatives that can minimize to a great extent the use of animals in the area of drug development and testing. The three R's are as follows:

## Refinement

To minimize the incidence or severity of inhumane procedures that may have to be applied unavoidably to animals in certain experiments. Any modification of a procedure that decreases potential pain or distress, or uses animals lower in phylogenetic category is acceptable.

The examples of refinement are:

1. Non-invasive monitoring, such as MRI.
2. Implantable monitoring device, such as recording of B.P.
3. Humane end points.

## Reduction

A reduction in the number of animals to be used in an experiment. For example, in the past, large numbers of animals were being used for the regulatory requirement of vaccine quality control. Following the principle of 3R's, there has been a significant reduction in the animal use. Strict adherence to the principle of 3R's in the vaccine quality control might eventually result in the total elimination of the animal use.

## Replacement

Substitution of insentient material in place of conscious higher animals. An experiment should not be performed on an animal if another scientifically satisfactory non-animal method of obtaining the result sought is available.

Replacement may be relative where animals are still required to provide cell, tissue, or organ, but experiments are conducted *in vitro* on perfused organs, tissue slices, tissue culture and on cellular and subcellular fractions.

The drug industry uses *in vitro* and other short-term non-mammalian tests for product development and drug discovery.

Some of the alternative non-animal tests are as follows:

#### A. *In vitro* pyrogen test

A number of alternative cellular assays have been developed, such as *Limulus* amoebocyte lysate (LAL) test, monocyte activation test (MAT), etc. replacing the animal rabbit pyrogen test.

All the test-systems are based on the response of human leukocytes (primarily monocytes) that release inflammatory mediators (endogenous pyrogens) in response to pyrogenic contamination (exogenous pyrogens).

The principle of the LAL test is that the lipopolysaccharides (LPS) cause extracellular coagulation of the blood (haemolymph) of the horseshoe crab *Limulus polyphemus* (Levin and Bang, 1964). The LAL test though more sensitive than the rabbit pyrogen test, it gives false negative results with certain products, likely to overestimate the pyrogen content of other products, and does not detect pyrogens other than the bacterial endotoxins (Gram-positive endotoxins), viruses and fungi.

The monocyte activation test (MAT) uses human mononuclear cells (e.g. monocytes) obtained from human volunteers or from the blood bank. This test detects pro-inflammatory and pyrogenic contaminants not always detected in the rabbit pyrogen test or in the LAL test.

#### B. Embryonic stem cell test (EST)

This is used for the detection of any embryonic toxicity. The embryonic stem cells develop spontaneously into contracting myocardium. The different end points of prenatal differentiation used in the mouse EST are as follows:

1. Inhibition of differentiation of the embryonic stem (ES) cells into cardiomyocytes
2. Cytotoxic effects on the ES cells
3. Cytotoxic effect on 3T3 fibroblasts

In the Embryonic Stem Cell Test (EST), the capacity of the stem cells (rodent cell line D3) to develop into specialized contracting heart cells *in vitro* within 10 days are used to assess the embryotoxic potential of the test compound by light microscopic evaluation, or with more objective molecular endpoints.

*In vitro* metabolism studies using human microsomal enzymes or cell lines provide information on whether a non-toxic chemical is likely to be metabolized to a toxic form, or *vice versa*.

A positive result in the EST should be regarded as sufficient evidence of embryotoxicity to classify a chemical as likely to be hazardous for development and reproduction. Negative results could be subject to further mechanistic assessments *in vitro*, if necessary.

### C. Local lymph node assay (LLNA) for skin sensitization

The basic principle underlying the LLNA is that when a test compound is applied on the skin, it is considered as a sensitizer when the lymph node draining the site of chemical application reveals a primary proliferation of lymphocytes as measured by the radioactive labeling. This proliferation is proportional to the dose applied that provides a simple means of obtaining an objective, quantitative measurement of sensitization. The LLNA assesses this proliferation in the form of a dose-response relationship in which the proliferation in the test groups is compared to that in the vehicle treated controls. The ratio of the proliferation in the treated groups to that in the vehicular controls is the *Stimulation Index*. The index must be at least three before the test substance can be further evaluated as a potential skin sensitizer.

### D. Clinical skin patch test on human volunteers (Basketter et al.1997)

Clinical patch tests offer the benefit of being directly relevant to the humans. Before a chemical is considered for a clinical patch test, it should be confirmed that it is not corrosive using the non-animal *in vitro* test. The chemicals that appear to be non-irritating and are negative in *in vitro* mutagenicity studies can be used in clinical patch test to confirm their non-irritancy. In most cases, properly conducted human patch tests (positive or negative response) are an acceptable alternative to the animal testing for skin irritation or skin sensitization.

### E. Neutral red uptake (NRU) assay

The NRU assay has been developed as an *in vitro* alternative (e.g. normal human epidermal keratinocytes (NHEK), balb/c 3T3 mouse fibroblasts and SIRC cell line derived from rabbit cornea) to the Draize rabbit eye test for the screening of chemicals for eye irritation potential (Harbell et al.,1997).. The NRU assay measures the ability of a test substance to inhibit the uptake of neutral red dye, a marker of cell viability. The neutral red penetrates cell membranes and accumulates intracellularly in lysosomes. Alterations of the cell surface or sensitive lysosomal membrane result in a decreased uptake of the neutral red. The NRU assay has been conducted on primary cell cultures as well as on established cell lines, e.g. Chinese hamster V79, CHO, 3T3 Balb/c and rabbit corneal cells (SIRC line). The concentration of test substance producing 50 per cent inhibition of neutral red uptake in comparison to control samples is obtained by extrapolation from the dose-response curves. This so-called NRU50 or IC50 value serves as toxicological endpoint.

### F. Carcinogenicity test

A substance's potential to induce carcinogenicity through non-genotoxic mechanisms can be assessed *in vitro* by means of cell transformation assays. Among the most promising examples are the Balb/c 3T3 Assay (Kajiwara and Ajimi, 2003)

and Syrian Hamster Embryo (SHE) assay (Englehardt *et al.*, 2004). A recent collaborative study by the International Life Sciences Institute (ILSI) found that these assays could be performed in different laboratories with a high level of reproducibility, while exhibiting a high level of predictivity. The SHE assay, in particular, was found to be sensitive to a wide range of both genotoxic and non-genotoxic carcinogens with 96 percent of known human carcinogens being detected. This assay is considerably faster (taking six weeks) and less expensive than the rodent bioassay and transgenic mouse models, and involves substantially fewer animals (maximum eight embryos against 800 or more animals in a standard bioassay).

### G. Acute toxicity test

Based on the premise that the actions of chemicals that produce toxicity do so at the cellular level (Grisham and Smith, 1984), many non-specific cell toxicity tests have been developed as potential replacements to acute lethality tests on animals. A combination of three such tests and a simple mathematical calculation was found to be significantly more predictive of human lethal doses for 50 chemicals than predictions based on rat and mouse LD50 values (Ekwall *et al.*, 1998). Regulatory guidance and recommended *in vitro* study protocols have since been published for use with normal human keratinocytes (NHK) and other standardized cell lines. A metabolic component can be incorporated into the assessment by means of *in vitro* metabolism studies, e.g. using human microsomal enzymes. In addition, there are several computer software packages for predicting acute toxicity from the chemical structure of the test compounds.

### H. Repeated dose toxicity test

The absorption, distribution, metabolism and elimination of a chemical by biological systems are critical in determining the nature and degree of toxicity that may result. Computerized biokinetic modeling is already widely used as a means of predicting the distribution of a chemical among the various organs and tissues of the body (Connell *et al.*, 1993), and by extension targeting for organ-specific toxicity (e.g. brain, liver, kidneys, etc.). Such predictions can then be verified quantitatively using cell cultures of these specialized tissues (Belamont *et al.*, 1999; Hartung *et al.*, 1999; Sheers *et al.*, 2001) thus effectively replacing the repeated dose study in animals with a combination of computerized biokinetic modeling and organ-specific *in vitro* assays

### I. Developmental neurotoxicity test (DNT) (Lein, 2007)

The procedures that will reduce the cost and the number of animals used, facilitate screening of large numbers of chemicals, and provide data for prioritization of chemicals for further targeted testing animals are as follows:

## Alternatives to Animal Experiments

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## CHAPTER 29

1. Use of alternative species (non-mammalian) with conserved neuro-developmental process.
2. Use of *in vitro* cell culture models
3. Development of *in silico* model

*In vitro* models are applied based on the key events of brain development that are conserved across the species, such as cell proliferation, migration, differentiation, survival and apoptosis, synaptogenesis, myelination, and axonal and dendritic outgrowth.

### Common sources of tissue for *in vitro* models

1. Avian – chick embryos
2. Rodents – rats and mice (wild type and transgenic):  
embryonic, post-natal and adult
3. Human – neural progenitor cells from aborted foetuses  
stem cell lines  
cord blood derived stem cells

### Types of *in vitro* systems – cell culture:

1. Cell lines
2. Primary culture
3. Culture with heterogeneous cell interactions

### Advantages of In Vitro Studies

1. Small quantity of test substance is needed
2. Experiment done under controlled condition
3. Result is obtained quickly
4. No major infrastructure is required

### Disadvantages of In Vitro Studies

1. *In vivo* dose-response not available
2. No systemic effect could be studied
3. Organ specificity lacking
4. Chronic and long-term effects could not be studied
5. Transportation of material not easy

### Enhanced Predictivity of the Drug Under Investigation for Human Use

1. Use of human based *in vitro* experimental system that gives human-specific data.
2. Use of relevant non-human *in vivo* model yielding key *in vivo* parameters.
3. Combination of *in vitro* and *in vivo* observations results in an enhanced predictivity of human usage of the drug under study.



A good *in vitro* test should ensure specificity, sensitivity, reliability (reproducibility), accuracy and predictivity.

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# Clinical Research

Clinical research is the scientific study that determines the safety and effectiveness of medications used in the diagnosis, treatment, prevention, or relief of symptoms in man. Pre-clinical studies, conducted on animals prior to clinical trials on humans, play a vital role in the drug discovery. These include a wide range of studies in a variety of systems such as *in vivo* animal models, isolated perfused liver, kidney, intestine, hind limb and heart, studies on animal and human liver microsomes, bioavailability and pharmacokinetic studies, metabolism in human liver microsomes, studies on cytochrome P450 enzyme models, assessment of potential for metabolic drug – drug interactions, *in silico* modeling, etc. Based on the results of the animal experiments the mechanism of action, potential therapeutic application, approximate dose range and possible toxicities are first established for a compound under investigation. The results are then translated in the humans in a cautious manner that forms the basis of clinical research. It deals with the drug dosage, pharmacokinetics, pharmacodynamics, pharmacogenetics and pharmacogenomics, factors affecting drug response and finally the toxicities and side effects. Pharmacogenetic studies identify the genetic factors responsible for either toxicity or failure of the drug under study. Drug toxicity occurs mainly due to its increased plasma level as a result of poor metabolizing capacity owing to genetic polymorphisms. In clinical trials pharmacogenomics play an important role in the assessment of several determinants of drug responses. The drug designed with pharmacogenomic support has a predetermined efficacy status in a sense that the chance of it failing in preclinical and clinical studies is minimum.

The fundamental principle of good clinical research is that the interest of science and society should never take precedence over considerations related to the well-being of the human subject under study. Good clinical practice (GCP) is a set of guide lines for biomedical studies that encompasses the design, conduct, termination, audit, analysis, reporting and documentation of the studies involving human subjects. It aims to ensure that the studies are scientifically and ethically sound, and that the clinical results of the pharmaceutical substances under study are properly documented. The guidelines seek to establish two cardinal principles: (a) protection of the rights of human subjects, and (b) authenticity of the data generated. These guidelines should be followed in carrying out all biomedical studies during all the stages of the drug development, prior and subsequent to the product registration.

The clinical research is conducted in four phases:

*Phase 1* – The primary objectives of this phase are: (i) to establish safe dose

range of the compound under investigation in healthy human subjects, and (ii) to provide sufficient pharmacokinetic as well as pharmacodynamic data upon which the subsequent clinical trials are based. In this phase, the compound already tested in the laboratory and in animals (preclinical phase) with satisfactory results is given to a small number (20 to 50) of healthy volunteers informed and agreed for the study. One of the most controversial areas in clinical research is the choice of the initial human dose. Dollery suggested that one or two per cent of the dose predicted from the animal data can be used. Pitts suggested that taking following factors into account a starting dose can be selected low enough to avoid unnecessary risk in humans: (i) theoretical maximum dose in man (assuming 70 kg as an average weight) is calculated as one-fifth or one-tenth of the maximum tolerated dose in the most sensitive species, (ii) an estimate of probable daily human dose from the animal study, and (iii) prior human experience with agents of a similar or related chemical class.

*Phase 2* – The major objectives of this phase are: (i) to provide detailed pharmacokinetic data, (ii) to establish clinical efficacy and the incidence of side effects and adverse drug reaction, if any, and (iii) to find out the most appropriate dosage schedule. Initially a small number of informed and consented patients are employed to determine the potential usefulness of the compound under test. Before involving a larger number of patients, a review of the results obtained in animal studies is made regarding the acute and chronic toxicities, and any effect on reproduction and fertility. If the compound found to be safe from these studies, a larger number of patients (100 – 300) are employed for a longer period to determine the final dosage form, and more data on metabolic activity.

*Phase 3* – During this phase the compound under investigation is tried out in a very large number of patients (1000 – 5000) in order to determine its efficacy and safety. By employing a 'double blind' method, where neither the patient nor the clinician knows which treatment (a placebo or a standard drug or the compound under investigation) is being given to whom. It is, however, unethical to withdraw an established drug in critically ill patients for the sake of clinical trial. At the end of this phase, if the compound found safe and effective, a product license is issued for marketing.

*Phase 4* – Once the compound is marketed, a careful study is made on the patients by the participating clinicians regarding the drug utilization pattern, additional efficacy, if any, or toxicity on long term use of the drug (pharmacovigilance).

The discovery, development and commercialisation of a medicine is a complex and time consuming process, which on the average, spreads between 12 to 14 years. Further, out of five thousand compounds evaluated initially, only five of them enter clinical trials. Of these five compounds only one gets approved for marketing

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# Molecular Cell Biology

## SOME BASIC PRINCIPLES

William Astbury in 1945 defined molecular biology as the study of the chemical and physical structure of biological macromolecules. The aim of the study is to understand the molecular basis of all biological phenomena. Simple systems such as bacteria, bacteriophages (bacterial viruses), yeast and fruit flies yield information about basic biological processes more readily than the animal cells.

There exist two types of organisms – *prokaryotes* and *eukaryotes*. Bacteria belong to unicellular prokaryotes while most organisms belong to multicellular eukaryotes. Fungi and protozoa, however, belong to unicellular eukaryotes. Prokaryotic cells are smaller than eukaryotic cells, and have simpler structure without any inner cell membranes. A eukaryotic cell has a nucleus that is separated from the rest of the cell by a membrane. The nucleus contains chromosomes, the carrier of the genetic material DNA. In prokaryotic cell the genetic material DNA is present throughout the cell mostly free of structural protein. The genetic material is RNA in some animal viruses and in a few bacteriophages. Within the eukaryotic cells there are membrane-enclosed compartments (organelles) like centrioles, lysosomes, Golgi complexes, endoplasmic reticulum, mitochondria, ribosomes etc., each responsible for a particular biological process. Mitochondria are specialized for energy production (respiration); lysosomes degrade worn-out cell constituents and foreign materials present in the cell; Golgi complexes direct membrane constituents to appropriate places in the cell; endoplasmic reticulum (a network of membranes) where glycoproteins and lipids are synthesized; and ribosomes where protein synthesis takes place. Organization, the form of genetic material, and the mechanism of protein synthesis, and regulation of gene expression are different in the two types of cell, and yet many biological processes are highly conserved from bacteria to man making the use of simple, experimentally tractable organisms useful in the study of such processes. The essential properties of most living cells are their ability to grow in an appropriate environment, and to undergo cell division with some exceptions, like neurons having only an initial growth phase. Prokaryotes, particularly bacteria multiply freely. Multi-cellular organisms begin life as a single cell (zygote) as a result of union of a male and a female sex cell (gametes). This single cell has to grow, divide and differentiate into different cell types to produce different types of tissues and organs in higher eukaryotes. However, the cell division and differentiation need to be controlled.

Cells consist of two basic types of molecules that control life:

1. *Small molecules* like water molecules, sugars, fatty acids, amino acids and nucleotides that serve as building blocks of the macromolecules. In addition, they have independent roles, such as signal transmission, or a source of energy for a cell to function. Biological membranes are mostly fatty acids into which macromolecules are embedded.
2. *Macromolecules* like proteins, DNA and RNA. Proteins perform two functions, one, structural, serving as the organism's basic building blocks, and two, functional, such as enzymes that catalyze a number of biochemical reactions. Transmembrane proteins are responsible for the maintenance of the cellular environment, regulating the cell volume, extraction and concentration of small molecules from the extracellular environment, and generation of ionic gradients like sodium/potassium pump essential for muscle and nerve cell functions. DNA, the main information carrier molecule in a cell, is either single or double stranded. A single stranded DNA molecule (polynucleotide) is a chain of small molecules (nucleotides) or bases, which are of two types, purines: adenine (A) and guanine (G), and pyrimidines: cytosine (C) and thymine (T). Different nucleotides are linked together in different order or in different sequence to form polynucleotides of different lengths. The two ends of this DNA molecule are chemically different and are marked as 5' on left and 3' on right. Specific pairs of nucleotides form weak bonds between themselves, 'A' binding to 'T' with two hydrogen bonds, and 'C' binding to 'G' with three hydrogen bonds. The A-T and C-G pairs are called *base-pairs* (bp) in terms of which the length of a DNA molecule is usually measured. Two complementary polynucleotide chains form a stable structure resembling a helix (DNA double helix) that serves as information storage. More bonds make the molecule more stable. The number of links needed to keep the double helix together depends on the temperature and other environmental factors. A single strand of the DNA molecule carries the information to be passed on to the next generation. DNA that is no longer in the helical form is said to be *denatured*. Molecular machinery in cells keeps both DNA strands intact and complementary by repairing a damaged strand using the second template. If the damaged DNA caused by environmental factors like radiation is not repaired the result can be cell death. RNA usually is single stranded having the same bases as DNA except that in place of thymine (T) it has uracil (U). RNA has different forms performing different functions, such as a messenger RNA (mRNA) that is complementary to one strand of DNA, and conveys information encoded in DNA to the translation machinery in the cytoplasm; a transfer RNA (tRNA) that transfers amino acids as per the mRNA coding; and a ribosomal RNA (rRNA) that is involved in protein synthesis.

## GENES AND PROTEIN SYNTHESIS

The whole process of transfer of genetic information from DNA to RNA leading to protein synthesis takes place in three stages:

1. *Transcription*: in this phase, two-stranded DNA double helix is first unwound, and one strand of DNA molecule is then copied into a complementary pre-messenger RNA (pre-mRNA) by the protein complex RNA polymerase II. Steroid hormones that are known to be major regulators of transcription in higher animals get bound in some cases to receptor proteins, which in turn activate transcription via sequence-specific binding to enhancer elements.
2. *Splicing*: removes some stretches of the pre-mRNA known as *introns*; the remaining sections called *exons* are then joined together producing mRNA. Exons are the part of the gene that code for proteins and they are interspersed with non-coding introns that are to be removed by splicing. Prokaryote RNAs do not have introns and so no splicing takes place.
3. *Translation*: is the process of making proteins in ribosomes by joining together amino acids as encoded in the mRNA. A particular amino acid is being determined by a base-sequence of three adjacent nucleotides in mRNA known as *triplet or codon*. The transfer RNA (tRNA) molecules each carry one amino acid to the ribosome specifically recognizing one codon on the mRNA. The end of translation is the final part of gene expression and the final product is a protein.

The enzyme *reverse transcriptase* copies RNA into DNA (reverse process of DNA to RNA). The enzyme found in retroviruses is used to synthesize a strand of DNA complementary to each viral RNA molecule. This enzyme can polymerize deoxynucleoside triphosphates into a complementary DNA strand using an RNA molecule as template. Like other DNA polymerases, reverse transcriptase can add nucleotides only to the 3' end of a preexisting primer base paired to the template.

## GENOME AND GENOMICS

*Genome* is the total genetic information carried by a cell or organism comprising chromosomal DNA that is in abundance and mitochondrial DNA. In a typical cell there are one or several long double stranded DNA organized as chromosomes. A human has 23 pairs of chromosomes. The number and size of the chromosomes are the same in all cells of an organism, but vary among different types of organisms. All organisms have genomes that encode almost all the hereditary information of the organism. All cells in an organism contain identical genomes as the result of DNA replication at each cell division. Genomes contain genes most of which encode proteins. DNA is the carrier of genes that is passed from generation to generation by DNA replication.

*Genomics* is the study of genomes including nucleotide sequence, gene content,

organization, and gene number. It is a collection of methods by which the clones are sequenced. A genomic library contains at least one copy of all the sequences in an individual's genome. Changes in genomic DNA are known as *mutations*.

*DNA sequencing* is the determination of the four-letter sequence for a given DNA molecule.

### RECOMBINANT DNA TECHNOLOGY (GENETIC ENGINEERING)

The term *recombinant DNA* refers to a new combination of DNA molecules that are not found normally. Recombinant DNA technology encompasses more than mere joining of DNA molecules, and includes multiple methods to analyze and manipulate DNA. In DNA cloning, the DNA fragments of interest produced by *restriction enzyme* digestion are first inserted into *vector DNA* molecules forming recombinant DNA molecules *in vitro*. The recombinant DNA molecules are then introduced into host cells where they replicate producing large number of recombinant DNA molecules. All the descendants from single such cell known as *clone* carry the same recombinant DNA molecule. Clones are identical organisms, cells or molecules descended from a single ancestor. Cloning a gene involves producing many identical copies that can be used for numerous purposes including research into the structure and organization of a gene, and the commercial production of proteins such as insulin. A vector or carrier must be able to replicate; there must be some way of introducing vector DNA into a cell; and there must be some means of detecting its presence, preferably by plating test in petri dishes. The three most common types of vector having all the three properties are plasmids, *E. coli* phage  $\lambda$ , and viruses. Plasmids are small circular DNA molecules present in bacteria. Plasmids replicate more or less independent of chromosomal replication, and are carried from one generation to the next. Plasmids are important elements in modern DNA technology for two reasons; one, they can be easily isolated and transferred to other host cells merely by mixing purified plasmid DNA with the desired host cells in appropriate solutions, and two, by use of restriction enzymes any gene can be inserted into the plasmid DNA. Another enzyme *DNA ligase* can insert DNA restriction fragments into DNA molecules producing recombinant DNA. The recombinant DNA molecules then can be introduced into appropriate cells, most often bacterial cells. All the descendants from a single such cell, called *clone*, carry the same recombinant DNA molecule. Once a clone of cells bearing a desired segment of DNA is isolated, unlimited quantities of this DNA can be prepared. In addition, DNA sequences up to about 100-base long can now be chemically synthesized by entirely automated procedures. Recombinant DNA thus can be produced containing either natural DNA fragments resulting from restriction-enzyme cleavage or from any desired chemically synthesized mutant sequences.

*Isolation of plasmid DNA:* The presence of plasmid DNA in a single bacterial



colony can be detected by electrophoresis. A single colony is taken and lysed, and then subjected to gel electrophoresis. The bacterial chromosome being large in size cannot penetrate the gel, while the plasmid DNA can. The rate of electrophoretic movement of DNA molecules through a gel increases with decreasing molecular weight, so that plasmid DNA forms a narrow band at a position in the gel characteristic of its molecular weight. The band is visualized by staining the gel with ethidium bromide that binds tightly to the DNA and fluoresces under ultraviolet light. The molecular weight of the plasmid DNA can be calculated from the distance moved in a particular time interval relative to that for plasmid of known molecular weight. This screening technique is very useful in recombinant DNA technology. Plasmid DNA can also be detected in a cleared lysate by electrophoretic technique. A vector and a DNA fragment of interest are cleaved with a *restriction enzyme (endonuclease)* that recognizes a specific base sequence in a DNA molecule, and makes two cuts, one in each strand, generating 3'OH and 5'P termini. The joining is accomplished by annealing the complementary single stranded ends by the action of an enzyme *DNA ligase* that catalyzes the formation of a phosphodiester bond between free 5' and 3' ends.

*Denaturation of DNA:* During DNA replication, the unwinding and separation of DNA strands first take place (denaturation or melting). The denaturation can be induced experimentally by heating a solution of DNA. The thermal energy increases molecular movement leading to the breakage of the hydrogen bonds and other forces that stabilize the double helix, thus separating the strands. The absorption of ultraviolet (UV) light by the DNAs is routinely used to measure DNA concentration in a solution. Single-stranded DNA absorbs almost twice as much UV light as does the equivalent amount of native double-stranded DNA. Thus, the absorption of UV light increases with the increase in the denaturation of DNA.

*Renaturation of DNA:* Native DNA can be reformed (renaturation) by special treatment of denatured DNA in a solution.

*Hybridization:* In a solution of denatured DNA, the single strands freely mix so that during renaturation, strands join together producing a molecular mixing known as *hybridization*. Hybridization occurs in a sequence-specific manner rather than at random, that is, single DNA strands will renature preferentially with other single strands that have a complementary sequence based on the pairing of A with T and C with G.

*Analysis of DNAs by Southern blot hybridizations:* Southern blotting, developed by Edwin Southern, is one of the most widely used methods for detecting hybridization between complementary nucleic acid (DNA or RNA) molecules. The essential feature of this technique is the transfer of DNA molecules separated by gel electrophoresis to a sheet of DNA-binding material like nitrocellulose or nylon membrane. The DNA in the gel is denatured into single-stranded fragments by treatment with an alkaline solution prior to the transfer. After the transfer is complete, the DNA is immobilized on the membrane by drying. A radioactive

DNA (probe) containing the sequence of interest is then hybridized with the immobilized DNA on the membrane. The probe will form a double helix only with DNA molecules on the membrane that contain a nucleotide sequence complementary to the sequence of the probe. Excess probe is washed away and the washed membrane is exposed to X-ray film that detects the presence of the radioactivity in the bound probe. After the development of autoradiogram the dark bands show the position(s) of DNA sequences that have hybridized with the probe. Southern blotting permits a comparison between the restriction map of DNA isolated directly from an organism and the restriction map of cloned DNA. It is also used to map restriction sites in genomic DNA next to the sequence of a cloned DNA fragment.

*Analysis of RNAs by Northern blot hybridizations:* Northern blotting, humorously named, is used to detect a particular RNA in a mixture of RNAs. An RNA sample is denatured by the treatment with formaldehyde that prevents hydrogen bonding between base pairs ensuring that all the RNA molecules have an unfolded linear conformation. The individual RNAs are separated according to the size by gel electrophoresis, and transferred to a nitrocellulose filter to which the extended denatured RNAs adhere. The filter is then exposed to a labeled DNA probe and subjected to autoradiography.

*Analysis of proteins by Western blot techniques:* In this, proteins are electrophoretically separated and transferred to a cellulose or nylon membrane to which the protein is covalently linked. Western blots are used to detect DNA-binding proteins. Radioactive double-stranded DNA is used as a probe. The association of radioactivity with a protein band indicates that the particular protein is a DNA-binding protein. Western blotting is more typically used to identify specific proteins in a mixture by the use of antibodies. After the separated proteins are transferred to the nitrocellulose, the membrane is exposed to an antibody to the protein of interest. The bound antibody is then detected by a second antibody (an anti-immunoglobulin that is conjugated to an enzyme usually horseradish peroxidase) that is able to bind to the first antibody. The presence of the enzyme is then detected by addition of a substrate that forms either a colored product or a chemiluminescent product that will darken X-ray film.

### DETERMINATION OF BASE SEQUENCE OF DNA

*Maxam-Gilbert procedure:* Single-stranded DNA is subjected to several chemical treatments that cleave the DNA molecule and generate a family of short single-stranded fragments. The number of nucleotides in each fragment is determined by gel electrophoresis that can separate molecules whose lengths differ by only a single nucleotide. Several cleavage protocols are used, each of which is base-specific and provides the base sequence. The more commonly used method is that of Sanger, and is based on chain termination by incorporation of dideoxynucleotide analogs.

### POLYMERASE CHAIN REACTION (PCR)

An alternative to cloning, the polymerase chain reaction (PCR) can be used to directly amplify manifold a specific DNA sequence in a complex mixture when the ends of the sequence are known. PCR can be used to clone a given DNA sequence *in vitro* without using living cells. This method of amplifying rare sequences from a mixture has numerous applications in basic research, human genetic testing, and forensics.

### DNA MICROARRAYS (DNA CHIPS)

DNA microarrays consist of thousands of individual gene sequences bound to closely spaced regions on the surface of a glass microscopic slide. By hybridizing RNAs from different experimental samples to the microarray chip it is possible to compare levels of thousands of individual RNAs simultaneously. Coupling these methods with the results from genome sequencing projects allow researchers to analyze the complete transcriptional program of an organism during specific physiological response or developmental process.

### CLONING OF cDNAs PRODUCED FROM PURIFIED mRNAs

The *in vitro* synthesis of cDNA (complementary DNA) from purified mRNA and other RNA molecules using *reverse transcriptase* has become a very important tool in molecular genetics.

### SITE-DIRECTED MUTAGENESIS

A useful experimental technique where a designed mutation is introduced at a prescribed site within a gene of interest. The main aim of the technique is to alter one or more specific nucleotides within a gene so as to change a specific triplet codon. As a result of transcription and translation, the change causes the insertion of a 'mutant' amino acid into the protein encoded by the original gene. Such designed mutations are particularly useful in the study of the effects of specific mutations on both gene expression and protein function. The basic steps of the technique are as follows:

1. A single strand of DNA from a gene of interest is first isolated.
2. This is hybridized with a synthetic oligonucleotide containing an altered triplet so as to encode an amino acid of choice.
3. Following semiconservative replication, a different complementary base pair is present in one of the new duplexes.
4. Following transcription and translation, a designed mutant protein will be produced.

### RNA INTERFERENCE

Molecules that can specifically silence gene expression are powerful research tools. RNA interference is a method to decrease ("knock-down") the level of specific mRNAs by the use of short interfering RNAs (siRNAs). siRNA is one of the latest sequence-specific gene-silencing agents. When the siRNA that is complementary to a short stretch of the target mRNA is introduced into cells, the expression of the targeted mRNA but not other mRNAs is decreased.

### GENE KNOCKOUT

It is a technique for selectively inactivating a gene by replacing it with a mutant allele\* in an otherwise normal organism.

*Gene-knockout in mice:* Gene-targeted knockout mice are powerful experimental system for studying their development, behaviour, and physiology. The procedure for producing gene-targeted knockout mice involves the following steps:

1. Mutant alleles are introduced into embryonic stem (ES) cells by homologous recombination
2. ES cells containing a knockout mutation in one allele of the gene being studied are introduced into early mouse embryos. The resultant mice will be *chimeras\*\** containing tissues derived from both the transplanted ES cells and the host cells. These cells can contribute to both the germ cell and somatic cell populations.
3. In order to assess whether the mutation is incorporated into the germ line the chimeric mice are mated.
4. Mice, each heterozygous for the knockout mutation, are mated to produce homozygous knockout mice.

*Use of knockout mice to study human genetic diseases:* Gene knockout mice are model systems for studying inherited human diseases. These are powerful tools for investigating the nature of genetic diseases, the efficacy of different types of treatment, and for developing effective gene therapies to cure these often devastating diseases.

### APPLICATIONS OF GENETIC ENGINEERING

The techniques of molecular biology enable research workers to apply to the studies of muscle and nerve function, membrane structure, mode of action of antibiotics and other agents, cellular differentiation and development, immunology, etc. Some of the applications of genetic engineering are as follows:

*Production of drugs:* (1) developing organisms that would abundantly produce antibiotics thereby reducing production cost, (2) producing biologically active compounds in large amount such as somastatin or growth hormone and insulin,

\* One of a pair, or series, of alternative forms of a gene that occur at a given locus in a chromosome.

\*\* An animal or tissue composed of elements derived from genetically distinct individuals

(3) producing a pure material like antiviral agent  $\alpha$ -interferon that could be tested clinically, and (4) cloning of interleukin II, a substance that stimulates multiplication of certain cells in the immune system; this is being tested on patients with AIDS.

*Synthetic vaccines:* The viral antigen genes are first cloned in an E.coli plasmid. Animal cell lines that support the growth of vaccinia are then infected with both normal vaccinia, and the plasmid DNA containing the viral-antigen gene. Genetic recombination occurs in the infected cells and some progeny vaccinia particles are formed that contain the cloned gene, thus the foreign viral antigen in the vaccinia coat. Various procedures are used to isolate these vaccinia hybrids. A surface antigen of *P. falciparum* has also been placed in the vaccinia coat; this may lead to the development of antimalarial vaccine.

*Gene therapy:* Somatic cell gene therapy has been used in the treatment of human diseases. It involves three sequential steps: (1) removal of some of the patient's cells, (2) introduction of normal functional copies of the gene that is defective into these cells, and (3) reintroduction of the *transgenic*\* repaired cells into the patient.

Since bone marrow transplant technology is highly advanced, obtaining cells for gene transplants and re-implanting the modified cells in the bone marrow of the patient should be a routine.

\* A cloned gene that is introduced and suitably incorporated into an animal, and is passed on to successive generations.

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# The Cell Line Studies\*

It is relatively easy to screen extracts and other materials for cytotoxic effects through automated screening procedures used in industry as well as by the research organizations, like the National Cancer Institute, USA running 60 different cancer cell lines. In India the National Centre for Cell Science (NCCS), Pune, an autonomous institute of the Department of Biotechnology (DBT), Government of India has a cell repository from where the cell lines can be procured.

A few common methods for studying cytotoxic activity are described below:

*Cytotoxicity testing.* It is based on one or more mammalian cell lines in actively growing condition undergoing mitotic division. The cells are cultured in a microliter-well plate; the rate of multiplication and growth is measured by the intensity of a colour directly proportional to the number of cells present. Different types of experiments are employed, the most basic one being a comparison of the rate of proliferation of a cancer cell line after a specified period of time in the presence and in the absence of the test material. In order to assess selectivity different cancer cell lines are used. The addition of normal cell lines to any of these determines the selectivity between the cancer and the normal cell lines. This gives an indication of the potential use in clinical setting for which a selectivity of at least two orders of magnitude in the susceptible cancer cell lines is undertaken. For determination of the cytotoxic effect whether cytostatic or cytotoxic, two sets of identical cells are exposed to the test agent under identical conditions for the same period of time. At the end of the exposure period one set of cells is assayed, whilst in the other set the medium containing test material is replaced by the fresh medium. The cells are then incubated for a fixed period of time before the assay for the cell growth. If the test material has only cytostatic effect, the cells will grow and undergo mitosis in the fresh medium. On the other hand, if it has cytotoxic effect, no such growth of cells will be observed.

Two major techniques are used to assess the cell growth. The first one uses either 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) or 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5 carboxanilide sodium (XTT) salt. The MTT method was first introduced in 1986 by Cole (Cole, 1986) followed by the XTT method in the year 1988 (Scudiero et al., 1988). Both the reagents MTT and XTT are metabolically reduced by the mitochondria present in the viable cells to a coloured formazan product, the intensity of which is measured spectrophotometrically in a plate reader. The use of XTT is preferred since the

\* Contributed by J. R. Vedasiromoni

formazan produced is soluble in water, whereas the solubilisation step is required if MTT is used (Skehan et al., 1996). However, many cell lines are not so efficient in reducing XTT compared to MTT; but with the addition of phenazine methosulphate (PMS) the reduction becomes much better (Scudiero et al., 1988). The second major technique preferred in testing cytotoxicity is the sulpho-rhodamine B (SRB) assay. This is based on the uptake of the negatively charged pink aminozanthine dye (SRB) by the basic amino acids present in the cells. The greater the number of cells, the greater the amount of dye being taken up. After fixing, the cells are lysed releasing the dye that gives more intense colour and greater absorbance (Skehan et al., 1996). The SRB assay is simple, sensitive, reproducible and faster than the formazan assay. It gives better linearity, good signal-to-noise ratio, and has a stable end-point that does not require a time-sensitive measurement compared to the MTT or XTT assays (Fricker and Buckley, 1996, Keepers et al., 1991). However, both the formazan based and SRB assays are being followed in different laboratories.

*Determination of  $IC_{50}$ .* It is a measure of the effectiveness of a compound in inhibiting the biological or biochemical function (vide chapter 19). The cancer cells ( $1 \times 10^5$ ) are seeded in 96-well sterile plates, and treated with different concentrations of the test material. The cell growth inhibition studies are done by the trypan blue exclusion method based on the fact that the dye enters only into the dead but not into the viable cells (Sur et al., 1995). With the help of a haemocytometer the number of living cells is determined under the microscope. The  $IC_{50}$  value is determined either by cell count method or by cytotoxicity studies or by both.

Apart from cytotoxicity testing the other types of experiments are conducted to determine whether the decrease in the number of cells is due to necrosis or as a result of apoptosis:

*Fluorescence microscopic studies.* The changes in the membrane permeability and in nuclear integrity of the leukemic cells treated with the test material are observed under fluorescence microscope. The cells ( $1 \times 10^5$ ) are treated with the desired concentration of the test material; after 24 h the treated and the untreated cells are harvested separately, washed with PBS, and stained with acridine orange (100  $\mu\text{g}/\text{ml}$ ) and ethidium bromide (100  $\mu\text{g}/\text{ml}$ ) in equal proportion. The cells are then mounted on a slide and observed under the fluorescence microscope for any morphological changes, like apoptosis or necrosis.

*Confocal microscopic studies.* The leukemic cells ( $1 \times 10^5$ ) are treated with different concentrations of the test material; the untreated cells and the treated cells are harvested and washed with ice cold PBS. The cells are then stained with nuclear binding dyes propidium iodide and Hoescht 33342 separately for 5 min. After mounting on slides under the confocal laser scanning microscope, any difference in the nuclear morphology between the treated and the untreated cancer cells are observed.

*Detection of apoptosis by DNA fragmentation and agarose gel electrophoresis.* The leukemic cells treated with the test material are harvested and washed twice with

PBS. They are suspended in 500  $\mu$ l of lysis buffer (50mM Tris HCl pH 8.00, 10 mM EDTA, 0.5% SDS); 100  $\mu$ g/ml of proteinase K is added and incubated at 50°C for 1 h and at 37°C overnight. DNA extraction is carried out following the general phenol-chloroform extraction procedure (Herrmann et al.,1994) and kept overnight at -20°C. After centrifugation, DNA precipitates are washed with 70% ethanol, dried at room temperature, dissolved in TE buffer (pH 8.0) and kept overnight at 4°C. The isolated DNA precipitates are subjected to electrophoresis overnight at 20 V in 1% agarose gel; after staining with ethidium bromide the DNA fragmentation is observed under UV transilluminator.

*Detection of apoptosis/necrosis by DOT plot assay using flow-cytometry.* In order to investigate the type of cell death by the test material, flow-cytometric analysis is done by the DOT plot assay. The leukemic cells ( $1 \times 10^6$ ) are treated with the test material; the cells are pelleted down, centrifuged at 2000 rpm for 8 min at 4°C and washed with annexin V FITC binding buffer (10 mM HEPES, 140 mM NaCl and 2.5 mM  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  pH 7.4). After centrifuging again at 2000 rpm at 4°C, the cell pellets are dissolved in annexin V FIT binding buffer containing in addition propidium iodide. Flow-cytometric analysis is done after incubation for 15 min in dark at room temperature.

*Study of cell cycle arrest by flow-cytometric analysis.* The cells ( $1 \times 10^6$ ) are treated with the test material, washed with PBS, fixed with cold methanol adding drop by drop and kept at -20°C for 3 min. They are then suspended in cold PBS, and kept at 4°C for 90 min. The cells are pelleted down, dissolved in cold PBS, treated with RNase A for 30 min at 37°C, stained with propidium iodide (20  $\mu$ l from 50  $\mu$ g/ml soln.) and kept in dark for 15 min. The flow-cytometric analysis is carried out immediately to determine if there is any cell cycle arrest.

### Advantages and Disadvantages of Cell Line Studies

#### A. Advantages

- i) A large number of extracts and other materials can be screened for cytotoxic effects using throughput automated screening procedures
- ii) Compared to *in vivo* and *in vitro* studies very small concentration of the material is required
- iii) The results are obtained quickly

#### B. Disadvantages

- i) Sophisticated and expensive equipments, like fluorescence microscope, confocal microscope, flow-cytometer etc. under aseptic condition are required.
- ii) Results are inconsistent with water insoluble materials excepting when they are soluble in less than 1% DMSO solution.



- iii) Results obtained with the cell lines may not corroborate with those of the *in vivo* studies.

The cell line studies provide a quick indication regarding the efficacy of the test material as well as its probable mechanism of action. A number of test materials can be screened at a time. However, in order to confirm the results obtained by the cell line studies, both *in vitro* and *in vivo* studies have to be carried out.

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# Nanotechnology in Biomedical Science\*

Nanotechnology is a well-known term that has become popular during the last few years. It is an interdisciplinary science dealing with the study of nanoparticles, their synthesis and application in our 'day to day life. The present chapter gives an overall view on nanotechnology, its applications with special emphasis on the techniques involved in biomedical science. Nanoscience and nanotechnology are new frontiers of the present century that have merged the disciplines of physics, chemistry, engineering and medicine. The concept of nanotechnology in science was first introduced by the physicist Richard Feynman in his talk given at the American Physical Society meeting at Caltech in U.S.A. on December 29, 1959, where he said "there's plenty of room at the bottom". The term "nanotechnology" was first introduced in 1974 by Professor Norio Taniguchi of Tokyo with the following words: "Nano-technology mainly consists of the processing, separation, consolidation, and deformation of materials by one atom or by one molecule". Since then the technology has been developed further by renowned scientist Dr.K.Eric Drexler.

Nanotechnology got started in the early 1980s with two revolutionary discoveries, one the birth of cluster science, and the other the invention of the scanning tunnelling microscopy (STM) which took the technology further step ahead. Today, nanotechnology is being employed in various fields, such as computers, electronics, communication, energy production, medicine, agriculture, food industry, cosmetics etc. Research and development at the nano scale level is booming worldwide, bringing forward medical and biological applications to a completely new horizon. The day is not far that this technology will become a part of our day to day life and solve many of the medical problems. Nanotechnology is manipulation of matter at nano scale level i.e., one billionth of a meter. In fact, it is a scale and not a type of technology. Materials when manipulated at nano scale level exhibit unique electrical, optical, magnetic, thermodynamic, thermal, chemical, and mechanical properties compared to its regular properties at bulk form (Patra et al, 2008). These unique properties make them potential candidates to be used in the field of biomedical science. Hence, in a broader sense it is the branch of technology which generally deals with quantitative concepts and measurement of objects based on the use of specific size-dependent properties. Materials where at

\* Contributed by Antony Gomes, Aparna Gomes and Jayeeta Sen Gupta

least one dimension is less than approximately 100 Nano meters are referred to as "nanomaterial" exhibiting unique optical, magnetic, electrical, and other properties having great potentials in the field of electronics, medicine, etc.

Nano materials can be broadly classified into:

- A. *Carbon black*: Carbon nanotubes, graphene, fullerene, and nanofibers are new class of carbon materials at nano scale level showing significantly different property from other forms of carbon (Kamat, 2006).
- B. *Metals/Alloys*: Different metals and alloys like gold, copper, silver, platinum, iron oxide, magnesium, palladium, selenium, cadmium, nickel etc. can be synthesized at nano scale level to be used in the diagnosis and treatment of diseases.
- C. *Ceramics and Polymers*: Ceramic nanoparticles like silica, titanium, and alumina can be engineered to desired size and porosity for their use as drug vehicles. Polymeric nanoparticles are bio-degradable that can be fabricated in a wide range of sizes and varieties (Yih et al, 2006).

### Nano materials : Synthesis and Characterization Techniques

There are two different approaches for the synthesis of nanomaterial: one, the top-down-cutting away material approach, and the other bottom-up- building or assembling approach. The different types of nanoparticles synthesis are:

*Liquid-Phase Synthesis*: This kind of synthesis involves co-precipitation, sol-gel processing, micro-emulsions, hydro-thermal /solvo-thermal, microwave, sono-chemical, template and biomimetic syntheses.

*Gas-Phase Synthesis*: It is a well-known chemical manufacturing technique for varieties of nano particles. This type of synthesis involves homogeneous nucleation of gases including laser vaporisation of solids, sputtering, inert gas condensation, expansion-cooling, laser ablation of solids, electrospray systems and spray pyrolysis (nano powders).

*Vapour-Phase Synthesis*: This technique includes the use of solid precursors involving inert gas condensation, pulsed laser ablation, spark discharge generation, and ion sputtering.

There are methods using liquid or vapour precursors involving chemical vapour, laser pyrolysis/photo-thermal, thermal plasma, flame, and low temperature reactive syntheses, as well as spray and flame spray pyrolysis. Nanoparticles after synthesis have to be characterized to determine the physical qualities such as diameter, particle size distribution, etc. with the help following methods:

*Analytical Ultra-Centrifugation (AUC)*: A high-speed centrifuge machine using rotors capable of sedimentation of colloidal and other small particles determines the size of such particles as well as weights of large molecules.

*UV-vis spectroscopy*: It includes a radiation source (visible/UV), filters (monochromators), sample cells/containers, and detectors. It is used in analytical chemistry for the quantitative determination of different types of analytes, such as

transition metal ions, highly conjugated organic compounds, conjugated nano compounds, biological macromolecules and nanoparticles. It is one of the important tools of nanoparticle characterization giving an overall picture of the size of the nanoparticles.

*Dynamic light scattering (DLS)*: It is also known as 'photon correlation spectroscopy' a common technology used to measure the size of nanoparticles, colloids, and molecules dispersed or dissolved in a liquid. The principle involves measuring the Brownian motion of particles or molecules in suspension that causes laser light to be scattered at different intensities. The intensity fluctuations are analysed to yield the velocity of the Brownian motion and thereby the particle size using the Stokes-Einstein relationship. By this technique the hydrodynamic diameter of the particles are recorded.

*Atomic Force Microscopy (AFM)*: This is one of the most promising tools that provide information on bio-recognition processes at single molecule resolution. AFM was developed to overcome the certain limitations of (STM). AFM measures forces acting between biomolecules undergoing bio-recognition process with pico Newton sensitivity in near-physiological conditions and without any labelling making it of great value in bio-nano technological analysis. In fact, it is capable of providing detailed information about the kinetics and thermodynamics of a single pair of interacting bio-molecules, offering the possibility to elucidate non-conventional aspects of bio-recognition processes, transient phenomena, conformational changes and molecular heterogeneity. AFM has been essentially used for studying surface interactions by means of force-distance measurement. AFM has the capability in imaging almost any type of surface, including polymers, ceramics, composites, glass, and biological samples.

*Transmission Electron Microscopy (TEM)*: Transmission electron microscopy provides information regarding morphologic, compositional and crystallographic constitution of the samples utilizing energetic electrons. It is capable of producing high-resolution, two-dimensional images, thus being utilized in all areas of biological and biomedical investigations. TEM images are formed using transmitted electrons (instead of the visible light) which are capable of producing magnification details up to 1,000,000X with resolution better than  $10\text{\AA}$ . Even the elemental composition of the sample with high spatial resolution can be determined. Due to its high resolution and magnification it is capable of imaging nanoparticles.

*Fourier Transform Infrared spectroscopy (FTIR)*: This includes an infrared source, interferometer, sample, detector and a computer. IR radiation is passed through the sample in which some of it is absorbed and some transmitted. The resulting spectrum represents the molecular absorption and transmission creating a molecular fingerprint of the sample. Like fingerprint, every molecular structure produces a unique infrared spectrum based on the compound analysed and characterized. This basic technique is an important tool to characterize nanoparticles in conjugations and combinations with other compounds. In fact, it can be used to

identify as well as to determine the quality or consistency of the sample, and, the amount of mixtures in the sample.

Major innovations in nanotechnology have brought forward the possibilities of its application in the field of biomedical science. Nanotechnology provides tools to enable us to learn precisely how cells and organs work. A combination of this new technology with the other existing ones has made it possible to reveal new frontiers in the fields of biology, medicine and biotechnology. The application of nanotechnology in medicine known as nano medicine mainly concerns the use of precisely engineered particles to develop novel therapeutic and diagnostic modalities. Nano materials are also applied in different fields of biology such as fluorescent biological labels, drug and gene delivery, bio-detection of pathogens, detection of proteins, probing of DNA structure, tissue engineering, tumour destruction via heating (hyperthermia), separation and purification of biological molecules and cells, MRI contrast enhancement, phagokinetic studies, cancer therapy, medical diagnostic tools and therapy. Medical nanotechnology is a combination of nano electronic biosensors, nanomaterial, and molecular nanotechnology leading to molecular manufacturing. It holds promise in providing economical yet quality healthcare requirements.

### Imaging

Inherited properties of nanomaterial, like superior photo stability, narrow range of emission, broad excitation wavelength, luminescence, surface Plasmon resonance, and multiple possibilities of modification, make them excellent candidates as bio-markers, sensors, and drug targeting agents. Quantum dots are highly light absorbing luminescent semiconductor nano crystals that are widely used in optical imaging. Conjugating particle surface with biomolecules, allowing cell targeting using quantum dots, is another development in nano-biomedical science. In fact, quantum dots are employed in the fluorescence resonance energy transfer analysis (FRET), gene technology, fluorescent labelling of cellular proteins, cell tracking, pathogen and toxin detection, and *in vivo* animal imaging. Advanced nanotechnology allows the manipulation of biological materials in a controlled way enabling integration with nanostructures. Manipulation systems in 2D include scanning tunnelling microscope (STM), atomic force microscope (AFM), scanning electron microscope (SEM), and transmission electron microscope (TEM). 3D manipulation systems include micro-grippers and nano-tweezers, manipulation using magnetic field, acoustic forces, microfluidics, and di-electrophoresis. Many of these techniques can be used to manipulate and integrate biological samples to build biological machines for diagnosis, bio-sensing, and bioelectronics.

### Molecular Targeting and Drug Delivery

Nanoparticles can be used as a drug delivery system, as their nano-size allow them to penetrate physiological barriers like small capillaries into individual cells,

targeting drugs towards specific locations in the body sparing the normal cells. The main principle is the entrapment or encapsulation of the drug within the nanostructure that is not rejected by the immune system while the drug is released uniformly over a period of time. The encapsulated therapeutic agent could be DNA, plasmid DNA, proteins, peptides, low molecular weight compounds, anti-cancer, anti-viral, and anti-bacterial agents.

### Combination molecules – Bio-Nano conjugation

“Bio-Nano conjugation” is the merging of biological and non-biological systems at Nano level. It is the combination of functionalities of biomolecules and non-biologically derived molecular species for specific use such as biomarkers, biosensors, bio-imaging agents, and masking of immunogenic moieties and targeted drug delivery systems. These are tailored systems to operate at cellular level for the diagnosis and treatment of diseases. Examples of such bio-conjugates with nanoparticles are quantum dots as fluorescent biological labels, gold nanoparticle bio-conjugate based colorimetric assay; gold nano-shell polymer composite photo thermally triggered drug delivery system, core/shell fluorescent magnetic silica-coated composite nanoparticles, bio-conjugated silica and colloidal gold nanoparticles etc. Similarity in size range of nano-materials and biomolecules make these dwarf particles attractive for intercellular tagging, and ideal for bio-conjugation, such as antibody targeting of a contrast agent.

### Bio-analysis and Biosensors

Nanoparticles can be used in a variety of bio-analytical formats in the form of quantification tags, such as optical detection of quantum dots, and the electrochemical detection of metallic nanoparticles, encoded nanoparticles as substrates for multiplexed bioassays, such as striped metallic nanoparticles. that leverage signal transduction. In colloidal gold-based aggregation assays, the functional nanoparticles exploit their specific physical or chemical properties in order to carry out novel functions, such as the catalysis of a biological reaction (Penn et al, 2003). The unique physical and chemical properties exhibited by materials at nano scale level also makes them very promising for designing efficient sensing devices, especially electrochemical sensors and biosensors. Nano materials in different structural forms like nano wires, nano rods, nano belts, nano rings, and nano crystals, provide high surface area, surface to volume ratio and interfacial properties compared to their counterpart at macroscopic size scale. This enabled the research workers to design different types of sensing devices. Many kinds of nanoparticles, such as metal, oxide and semiconductor nanoparticles have been used for constructing electrochemical sensors and biosensors. (Luo et al, 2006).

### Biomedical Applications of Nanotechnology

The integration of nanotechnology with biotechnology and medicine enables

the research workers to unveil the nature and working of the biological systems at nano scale perspective. Nanotechnology provides tools to interface living cells with engineered particulate matter, which enables sensors to be implanted within the human tissues to obtain vital information on biological processes and functions.

*Stem Cells Therapy:* These are powerful tools currently employed to treat injuries and tissue degeneration etc. However, they have their limitations that can be solved by merging nanotechnology with stem cell therapy. Nanoparticles in stem cell therapy involve magnetic nanoparticles and quantum dot-based applications which can track and guide transplanted stem cells and control functions of cellular signals. Nano systems have also find its application in the fields of atherosclerosis, thrombosis, and vascular biology (Wickline et al., 2006).

*Cancer Therapy:* Cancer nanotechnology is used to characterize the interaction of nano scale devices with cellular and molecular components specifically related to cancer diagnosis and therapy. Cancer nanotechnology holds promise in the field of cancer therapy since these particles can be modified to engineer vehicles with unique therapeutic properties, which because of their small size can penetrate tumours with a high-level specificity and target oriented therapy. The National Cancer Institute, USA has recognized the potential of nanotechnology providing a significant breakthrough in the diagnosis and treatment of cancer.

### Nano Toxicology

Owing to the increasing application in medical science these dwarf particles are liable to produce detrimental effects in human body as well as to the environment. Nanoparticles can potentially cause adverse effects on organs, tissues, and in cellular, subcellular and protein levels because of their unusual physicochemical properties, such as small size, solubility, shape, high surface area to volume ratio, chemical composition, crystallinity, electronic properties, surface structure reactivity and functional groups, inorganic or organic coatings, and aggregation behaviour. Sengupta et al., (2012) have recently reported toxicity of gold nanoparticles in the animal system. Spions group of metals which are being widely used for various biomedical applications, such as magnetic resonance imaging, targeted delivery of drugs or genes, and in hyperthermia may lead to severe biological hazards. Hence a complete understanding of the hazard of nanoparticles both in human body as well as in environment is absolutely essential.

### Safer Nanotechnology – Green synthesis

Though the physical and chemical are popular methods of nanoparticle synthesis, recent concepts indicate that the biogenic production of nanoparticles is a better option due to its eco-friendliness (Salam et al, 2012). Green synthesis refers to a method by which nanomaterials are produced without affecting the environment or human health. Different natural products, such as extracts of various plants, tea, coffee, banana, simple amino acids, as well as wine, table sugar,

glucose, bacteria, algae and fungi are used as reductant, and as capping agents to synthesize nanoparticles thereby reducing the hazards of nano toxicity.

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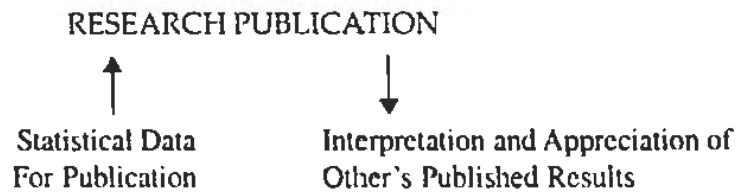
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# Biostatistical Methods

Statistics by definition is a science of numbers. Statistical method deals with the designing of experiment followed by collection, analysis, interpretation and presentation of data accompanying appropriate test of reliability. Statistics applied in biological science is known as biostatistics.

## WHY STATISTICS?



For any research publication in the field of biochemistry, microbiology, physiology, pharmacology, pharmaceutical sciences etc., incorporation of statistical data is a must in support of the conclusion drawn in the experiment. Thus, some knowledge of basic statistics is an essential requirement on the part of the investigator. One may argue by saying that in this computer age where software on statistics (SigmaStat, GraphPad, SPSS, etc.) are available that carry out all the statistical analysis of the data, where is the need to know about statistics. This concept is absolutely wrong because the computer will carry out whatever data are being fed as an input whether right or wrong. If the data of an experiment not designed properly are fed into the computer, the results obtained may not be dependable. Thus, the knowledge of proper designing an experiment is a prerequisite in order to draw a reliable conclusion from the results obtained in the experiment. Another reason why one should have some basic knowledge of statistics is in the interpretation and appreciation of other's published results based on the statistical data and to check its correctness.

Before we go into the experimental design and statistical analysis, it would be prudent to consider some of the elementary arithmetic that forms the foundation of any dependable quantitative study.

## SCIENCE OF NUMBERS

In pure mathematics the numbers 40 and 40.00 have the same meaning. But in the scientific work these numbers have different meaning. In science, the number 40 means that the measurement lays between 39.5 and 40.5, that is a difference of one, while 40.00 means that the measurement lies between 39.995 and 40.005, that

is a difference of 0.01. In other words, the number 40.00 is measured more accurately than the number 40.

**Significant Figures**

Significant figures (S.F.) are those that have practical meanings. The more the number of significant figures more towards the accuracy. Every digit is significant except zeros. Zeros are significant unless they are at the extreme right of a number and to the left of the decimal point, or at the extreme left of a number.

*Examples:* 125 (3 S.F.), 1250 (3 S.F.), 1205 (4 S.F.), 0.0125 (3 S.F.) and 1250.00 (6 S.F.).

Few biological measurements can be made with more than three significant digits; though up to six may reasonably be retained for intermediate calculations not more than four should normally be presented in the final results.

**Rounding off Numbers**

In rounding off the numbers, the accepted principle is to leave the last digit retained unchanged if the quantity rounded off is less than half a unit, but increase it by one unit if the quantity exceeds half.

*Examples:* The figures 39.3 are rounded off to 39, while 70.8 are rounded off to 71.

If the quantity rounded off amounts to exactly half, the convention is to leave unchanged the last digit retained if it is even, but to increase it by one unit if it is odd.

*Example:* The figures 87.5 rounded off to 88 while 58.5 are rounded off to 58.

Supposing, we are given certain numbers and asked to round them off to two significant figures, we have the following:

1425 rounded off to 1400; 1550 to 1600; 1268 to 1300; 1850 to 1800; 1996 to 2000.

The last answer 2000 is not correct since it has only one significant figure instead of two. The correct answer is  $2.0 \times 10^3$  that indicates that there are two significant figures. This type of representation of number is known as the "scientific or standard notation" very often used to indicate a concentration or dose of a substance, the expression of which otherwise is very unwieldy. This is done by writing the number with one figure to the left of the decimal point multiplied by the appropriate power of 10.

<i>Example:</i>	31200000	=	$3.12 \times 10^7$
	312	=	$3.12 \times 10^2$
	0.00312	=	$3.12 \times 10^{-3}$

**Accuracy of Measurements**

It is extremely easy to do arithmetic. Herein lies the difficulty. For instance, the average or mean of a group of individual values can be calculated to umpteen

places of decimals with astonishing ease, especially with the help of a calculator, and the result thus presented may look very accurate.

Suppose, we have measured the body weight of six rats in a balance that measures accurately up to 0.5 G. division and we have the values in G. as follows:

145.5      139.0      142.5      151.5      137.0      148.0

We get a mean value of 143.917 and present it as 143.92. Are we presenting correctly the mean value of the body weight of six rats? Depending on the accuracy of the measurement only as many significant figures should be reported as the correct value. The measured values have four significant figures. Hence, the mean value should be 143.9. But the balance can measure accurately up to 0.5 G. So the final correct mean value should be 144.0. To make a report more accurate than the actual measurement is a misrepresentation.

### MEASURE OF CENTRAL TENDENCY OR AVERAGE

An average is a typical value that represents a group of individual values in a simple and concise manner. It tells us the point about which the several values cluster. The sample mean is an unbiased estimate of the population mean. More the number of observations, nearer the true value of the population mean.

#### Arithmetic Mean or Mean

The terms average, mean and arithmetic mean are commonly used interchangeably. Mean (A.M.) summarizes the results in a single value. It is typical of the whole set. In successive samples from the same population the mean varies less than the individual values (Table 34.1).

Table 34.1

	75	80	80	88
	86	85	77	90
	68	75	76	110
	72	74	84	93
	85	80	73	72
Mean	77	79	78	90

Thus, it gives us confidence in using the mean as a final value.

The mean ( $\bar{x}$ ) is equal to the sum of the individual values (SX) divided by the number of individual items (N):  $\bar{x} = SX/N$

The mean can be treated further for statistical analysis. However, the disadvantage is that the mean is greatly distorted by extreme values and thus may not be typical in certain cases.

For example, the mortality rate per month in a particular year in a town in Europe during the epidemic of influenza was recorded as follows:

6    7    7    9    8    6    8    8    865    20    10    9

Mean (A.M.) is calculated as 80 which neither represents the 11 small values nor the single extreme value of 865.

**Median**

It is the calculated value of the middle item when the items are arranged in order of magnitude. When the items are in large numbers, the simple formula to locate the middle item is by the formula:  $(N + 1)/2$ .

For example, if there are 171 items, the middle item is  $(171 + 1)/2 = 86$ th item. When the items are in even number, the median is the arithmetic mean of the middle pair.

The median is the average of position while arithmetic mean is a calculated average. Median is affected by the number of items and not by the size of the individual items. Measurement of the individual items is not required but only of the middle item. It is used in the calculation of the median lethal dose (LD50) and in the median effective dose or concentration (ED50 or EC50) though not generally appreciated. Not distorted by extreme values as can be seen in the case of mortality rate due to the epidemic of influenza:

6 7 7 9 8 6 8 8 865 20 10 9  
 A.M. = 80                      Median = 7

Median 7 is more representative than the A.M. 80.

The median is not so generally familiar as the arithmetic mean nor is it amenable to statistical analysis.

**Geometric Mean**

The geometric mean (G.M.) is the Nth root of the product of N items:

$$G.M. = \sqrt[N]{(X_1 \cdot X_2 \cdot \dots \cdot X_n)}$$

For a large series of items, the computation is simplified by converting the original values into log values:

$$\text{Log G.M.} = (\log X_1 + \log X_2 + \dots + \log X_n) / N$$

Antilog of the coefficient gives the G.M. In other words, G.M. is the antilog of the arithmetic mean of log doses. Since so many dose-response curves are constructed and analyzed with the log dose, the G.M. is commonly used but not always appreciated.

G.M.'s of EC50 values and their 95% confidence limits are calculated in the following steps (Fleming, *et al.* 1972):

- i) EC 50 value is determined from the dose-response curve of each experiment.
- ii) Values are converted into log values.
- iii) Mean log EC 50 value is calculated.
- iv) Confidence limits (95%) are calculated.
- v) Converting the mean and the confidence limit values to their antilog values yield the G.M. and their 95% confidence limit.

G.M. is the appropriate average of quantities that follow a geometric progression

or an exponential law like population growth or bacterial growth. It is a calculated average like arithmetic mean dependent upon all the individual values. It is more typical average than the arithmetic mean since it is less affected by extreme values. The disadvantage is that it is not widely known average, relatively difficult to compute, and cannot be employed if any item is negative or zero.

**Mode**

The mode is a most commonly occurring value provided a sufficiently large number of items are available to give a smooth distribution. It is independent of extreme items. The mode like median is an average of position. In a small number of cases the mode may not exist as none of the value may be repeated.

**Harmonic Mean**

The harmonic mean (HM) is the reciprocal of the arithmetic mean of the reciprocals of the number of items (replicates) in each sample:

$HM = N / (1/k_1 + 1/k_2 + \dots + 1/k_n)$ , where k is the sample size (replicates) and N the number of items.

*Example:* The harmonic mean of four items say, 5 9 4 7 is computed as below:

$$\begin{aligned} HM &= 4 / (1/5 + 1/9 + 1/4 + 1/7) \\ &= 4 / (0.20 + 0.11 + 0.25 + 0.14) \\ &= 4 / 0.7 \\ &= 5.71 \end{aligned}$$

The harmonic mean is used in the Dunnett's test described later.

**SCATTER OR VARIATION**

The average or mean though a typical value it does not tell the whole story about the distribution of individual values around the mean as shown in Table 34.2.

Table 34.2

	119	122	51
	120	118	17
	121	124	387
	120	120	5
	120	116	140
Mean	120	120	120

As can be seen, although the means of all the three groups are identical, the variation amongst the individual values in the first group is slight, in the second group intermediate and in the third group wide. Hence, only the measurement of average is incomplete without some measure of variation of the individual values around the mean.

**Range**

Range is the difference between the lowest and the highest value. Though it is a simplest measure of variation, it is not dependable since it is based only on the two extreme values ignoring the variability amongst the other intermediate values.

**Mean Deviation**

Mean deviation is the arithmetic mean of the deviation of individual values from the mean ignoring the signs. It is better than the range but not ideal since signs are ignored. Mean deviation is not amenable to statistical analysis.

**Standard Deviation**

The most important measure of variation is the standard deviation, which is a special form of average deviation from the mean. It is the spread of the individual values around the mean in a sample. It is a typical deviation as much the same way that the mean is a typical observation. It is used widely since it is amenable to statistical analysis.

The standard deviation is calculated in the following reverse steps of the phrase "root-mean-square deviation":

1. Calculate the deviation of the individual values from their mean ( $d$ )
2. Square up  $d$  to get rid of signs ( $d^2$ )
3. Add up  $d^2$  to get "sum of squares of deviations" ( $Sd^2$ )
4. Divide  $Sd^2$  by  $(N-1)$  to get "mean square deviation" or "variance" ( $s^2$ )
5. Take the square root (reversing step 2) of the variance ( $s^2$ ) to get the standard deviation S.D. or  $s = \sqrt{s^2} = \sqrt{(Sd^2/N-1)}$ .

An alternative formula for calculating  $Sd^2$  is commonly employed bypassing steps 1 and 2 as follows:

$Sd^2 = SX^2 - (SX)^2/N$  where  $X$  is the individual value,  $S$  the sum and  $N$  the number of items.

Dividing the sum of squares ( $Sd^2$ ) by  $N-1$  (degrees of freedom) instead of by  $N$  needs some explanation. Our main interest is in the population variance that we estimate from large samples. Small samples of say, less than 30 items tend to under estimate the variance of the population since the range of the sample is likely to be much less than that of the population. The sample variance in such a situation would likely to fall short of that of the population if division of the sum of squares were by  $N$ . In order to counterbalance this, the division of the sum of squares is carried out by one less than the number of items that is by  $N-1$  rather than by  $N$ , thus giving a better estimate of the population variance.

Standard Deviation of the Mean or Standard Error

If a series of random samples are drawn from a population their means will vary from one to another though to a lesser degree compared to the variation of individual values in a sample (see Table 31.1). The standard error is the spread of the means around the mean of means of a number of samples. The greater the sample size, the smaller will be the standard error.

A simple way of finding out the standard error (S.E.) is to calculate the S.E. of just one sample. This S.E. is an estimate of the standard deviation (S.D.) that would be obtained from the means of a large number of samples drawn from the same population.

The standard error is obtained by dividing S.D by square root of the number of items in a sample (N):

$$S.E. = S.D./\sqrt{N}$$

Alternatively, S.E. can be obtained by dividing the variance ( $s^2$ ) by the number of items (N), and then extracting its square root:

$$S.E. = \sqrt{(s^2/N)}$$

Standard error varies directly with the standard deviation and inversely with the square root of the number of observations. In order to reduce the standard error to half, the number of observations has to be increased by four times. The standard error is used in the determination of confidence limits, in the Student's t test and in the analysis of variance.

*Determination of Standard Deviation and Standard Error*

Example : Atropine 1  $\mu$ g produced inhibition of methacholine induced gastric secretion in a rat measured continuously by recording in a pH meter. The same treatment is repeated four times and the results are presented in Table 34.3.

Table 34.3

<i>pH values</i>	<i>Square</i>
1.4	1.96
0.9	0.81
1.8	3.61
1.9	3.61
SX = 6.1	SX <sup>2</sup> = 9.99

Mean ( $\bar{x}$ ) = SX/N, where S is the sum, X the individual values and N the number of individuals values:  $\bar{x} = 6.1 / 4 = 1.52$

*Steps for calculation of standard deviation and standard error:*

1. Add up the individual values SX = 6.1
2. Square up the individual values and add them up SX<sup>2</sup> = 9.99

3. Find the sum of square of deviation from the mean  
 $Sd^2 = SX^2 - (SX)^2/N = 9.99 - (6.1)^2/4 = 0.69$
4. Divide  $Sd^2$  by  $N-1$  to get the variance  
 $s^2 = Sd^2 / N-1 = 0.69/3 = 0.23$
5. Extract square root of  $s^2$  or variance to get the standard deviation  
 S.D. or  $s = \sqrt{s^2} = \sqrt{(Sd^2/N-1)} = 0.48$
6. To get standard error (S.E.) either divide S.D. by  $\sqrt{N} = 0.48/2 = 0.24$  or extract square root of  $(s^2/N) = \sqrt{(0.23/4)} = 0.24$

### NORMAL DISTRIBUTION

Many biological characteristics conform to a so-called "normal" or "Gaussian" distribution that is always symmetrically bell-shaped, and unimodal having mean, median and mode all at the same point, and completely described by two parameters that is mean and the standard deviation (Fig. 34.1).

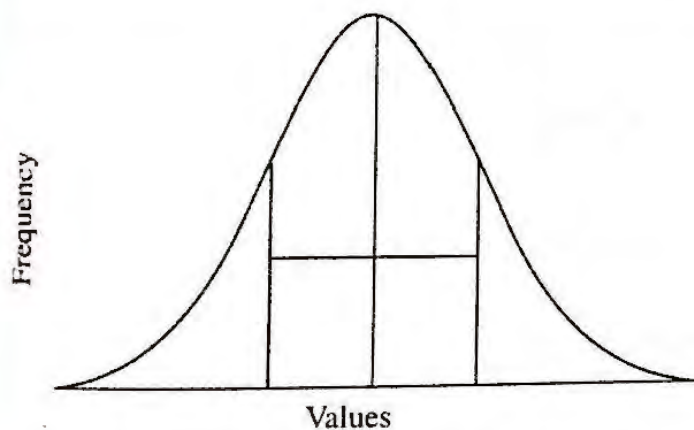


Fig. 34.1 Normal bell-shaped curve with lesser number of items at the extreme ends (cf. *t*-distribution curve)

When the data set follows normal distribution, we can estimate the spread of the data as approximately 95% falling within mean  $\pm 2$  S.D. The means of samples drawn from normal population are normally distributed. The means tend to be distributed normally even when the parent population is not normally distributed. Thus, the means have a greater role in

practical applications where the exact form of the sampled population is not known. As the sample size increases the shape of the sampling distribution approaches normal. However, it should be borne in mind that there is nothing abnormal about any observed distribution that does not follow the normal distribution. In such a case they should be treated as a-normal or non-normal rather than abnormal.

### *t* DISTRIBUTION

In the case of small samples containing less than 30 observations unlike the normal distribution with large samples, the ratio of the differences between the means of the samples to their S.E.s will not be normally distributed but in a rather different shaped curve known as the *t* distribution described by W.S. Gosset, a civil servant under the pseudonym "Student" (Fig.34.2).

The shape of the *t* distribution curve depends on the degrees of freedom (d.f.), the concept also developed by "Student". The *t* distribution has relatively more values in its tails (leptokurtic) than does the normal distribution (cf. normal



distribution curve). With very few d.f. the *t* distribution is very leptokurtic. As the d.f. increases the *t* distribution approaches the normal distribution.

### *t* VALUE

The quantity *t* is a ratio defined by the formula  $t = (\bar{x} - m)/S.E.$ , where  $\bar{x}$  is the sample mean and *m* the population mean and S.E. the standard error of the mean. We know  $\bar{x}$  and S.E. from our sample; we select an appropriate value of *t* corresponding to a particular probability level from the table of *t* distribution (see Appendix), and calculate the value of *m*, that is the population mean:

$$m = \bar{x} \pm t_{0.05} S.E.$$

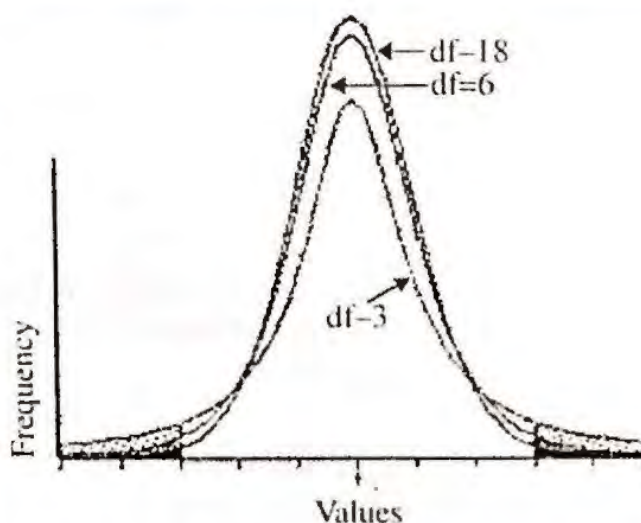


Fig 34.2. *t* distribution curve with more and more items at the extreme ends with less and less degree of freedom (cf. normal distribution curve)

### CONFIDENCE LIMITS OR FIDUCIAL INTERVALS

These describe the limits within which 95% of the mean values are likely to fall if determined in similar experiments. The value of *t* corresponding to a probability of 0.05 for the appropriate degree of freedom (*N* - 1) is read from the table of *t* distribution (see Appendix). By multiplying the value with the S.E. the 95% confidence limits for the population mean (*m*) are obtained as per the formula below:

$$m = \bar{x} \pm t_{0.05} S.E.$$

Lower value of  $m = \bar{x} - t_{0.05} S.E$  Higher value of  $m = \bar{x} + t_{0.05} S.E$

Thus, we may say that the population mean (*m*) lies within these limits in 95% of cases. The probability of the mischance by the sampling that may fall outside this limits is only 5% that is one in 20.

*Example:* Mean and S.E. of safety index of halothane in 10 mice were as follows:

Mean = 2.21                      S.E. = 0.15                       $t_{0.05}$  for 9 d.f. = 2.26

Confidence limits:

Lower value =  $2.21 - (2.26 \times 0.15) = 1.87$

Higher value =  $2.21 + (2.26 \times 0.15) = 2.55$

Thus, the mean is 2.21 and C.L. at 95% level is 1.87 to 2.55

### COEFFICIENT OF VARIATION

When the mean of two samples are almost similar, looking at their standard deviations (S.D. or *s*) we may be able to say which of the sample is more variable. If the means, on the other hand, are widely different it may not be easy to say which

of the sample is more variable. In such a case the variables can be compared by finding the relative standard deviation or coefficient of variation (C.V.) of each sample where the standard deviation of each sample is expressed as a fraction of the mean ( $s/\bar{x}$ ). The C.V. is usually expressed as a percentage of this fraction :

$$C.V. = (s/\bar{x}) \times 100.$$

Knowledge of C.V is useful both in planning as well as in evaluating experimental results.

*Determination of sample size that is required to give a reliable result :* The C.V. is utilized in estimating the sample size depending upon the results of the preliminary experiments showing no difference between the means of the two samples. The formula used for the purpose is derived from the equation we know already:

$\pm t = (\bar{x} - m)/S.E$ ; substituting 0 for m, that is no difference between the population mean and the calculated mean, and  $s/\sqrt{n}$  for S.E. we get the equation:

$$\pm t = \bar{x} / (s/\sqrt{n}) = \bar{x} \sqrt{n}/s$$

$$n = t^2 s^2 / \bar{x}^2 = t^2 CV^2$$

Thus, a sample size may be estimated for any specified probability (say 5%) of  $t$ , provided some idea about  $s$  and  $\bar{x}$  are available from the prior experiment.

*Evaluation of experimental results:* It is also a convenient way of comparing two variables independent of the units or the methods employed in the experiment.

*Example:* Comparison of the variability between the two methods of measurement of the rat-paw oedema (Table 34.4).

**Table 34.4**

Method	Mean	S.D.	C.V.
Harris & Spencer (1962)	0.60	0.23	38.3 %
Singh & Ghosh (1968)	0.78	0.15	19.2 %

C.V. values indicate that the method employed by Singh & Ghosh is less variable than that of Harris & Spencer.

It should be remembered that the coefficient of variation is informative and useful only if it is presented along with the values of  $\bar{x}$  and  $s$ .

### EXPERIMENTAL DESIGN

If an architect's plan (blue-print) is faulty then the construction of the building becomes defective notwithstanding the use of the best materials and labours. Similarly, if the design of an experiment is faulty, any statistical interpretation that makes the result conclusive must be faulty too. The experimental design and statistical procedure are interdependent, and one without the other makes any conclusion drawn from an experiment invalid. Hence, it is imperative that before

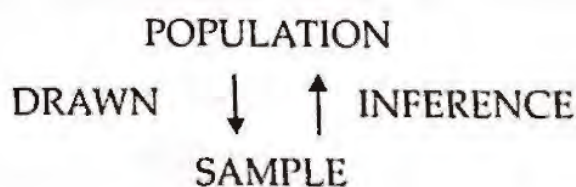
starting any experiment, proper designing is carried out, if necessary, with the help of the statistician in order to draw a valid conclusion from the results obtained. To make this collaboration successful the statistician should learn all he can about the problem in hand, and the experimenter all he can about the basic statistical approach.

### Population

In statistics the term population has a different meaning from the one used in ordinary speech. Besides human, animal or plant population the statisticians also speak of population of objects, events, procedures etc. In statistical term a population is an aggregate not only of creatures and plants but also of objects, events, procedures, things etc.

### Sample

Since a population contains too many individuals or items to study conveniently, an investigation is restricted to one or more samples drawn from it. Samplings are thus devices for learning about large masses that is population by observing a few individuals that is sample. We get from our sample statistic an estimate of the population parameter.



### Examples of Sampling

The physician makes an inference about the patient's blood through examination of a drop or a few ml of blood. A dozen rats may suffice to disclose useful facts concerning a population that runs into hundreds and thousands. A simple household example of sampling is to take a few grains of boiling rice and press them between the fingers in order to see if the whole quantity of rice in the pot has been cooked properly or not.

### Replication

The replication helps us in isolating response due to treatment from that due to chance variation that is the experimental error. The replication not only reduces the experimental error but also actually furnishes an estimate of the error that remains. The difference amongst the values in each group provides variation as a result of chance (within group variation), while the difference between the groups provide variation partly due to the chance and partly due to the effect of the treatment (between group variation). By isolating the within group variation from

the between group variation we get the effect due to the treatment. This is the basis of the *analysis of variance* that is considered later.

### Randomization

Inference about the population may not be true unless the samples are drawn in a random fashion. An important attribute of a sample is that every individual or item in the population from which the sample is drawn must have an equal chance of being included in it. In other words, there should be no favoritism or bias in the process of drawing samples. In an unbiased sample, as the number of item is increased the mean tends toward the population mean. To ensure un-biasness we have to adopt a method of randomization.

In randomization the selection of the subject is made by means of a process in which chance alone and not subjective biasness operates. This could be either by spinning a coin, or drawing cards, or more commonly the use of the table of random numbers (see Appendix). A sample so chosen is called a random sample.

*Table of random numbers:* The numbers in the table are arranged in blocks of five for easy reading. In practice, two consecutive numbers are taken together for allotment in a random fashion. For example, we want to conduct an experiment using three groups of rats each group comprising 10 rats. Three groups of rats are given different treatments including the control. Before starting the experiment we have to allot the rats in a random fashion to different groups. In the first group we allot rat No.1 to 10, in the second group rat No.11 to 20, and in the third group rat No. 21 to 30. In order to do this in a random fashion we start from any point in the table and then proceed in any direction allotting the rat to the particular group. Suppose we start at a point in row 14 and column 20-24 and proceed towards the right direction and we get the numbers 23 41 01 27 40 02 54 05 44 40 32 94 9 etc. We pick up the rats one by one and go on allotting to different groups as per the number in the table. For instance, we allot the first rat to the third group the number being 23. In the process, we ignore if the number allotted appears again and also any number greater than 30. The next rat we allot to the group one the number being 01 and so on till all the three groups have been completed. We have to skip off a large combination of numbers in the table being greater than 30. To overcome this the alternative method will be to divide the number by 30 if it is larger and take the remainder for allotment ignoring if remainder was zero. In the present series we divide 41 by 30 and allot the remainder 11 to the second group, and so on till the process is completed. We ignore if a particular number has been allotted already. Once this allotment process is over we treat one group as control, one treated with a standard drug and the third group treated with the compound under study.

*Latin square design:* In this design perfected by R.A.Fisher, the number of replicates (rows) is same as the number of treatments (columns). It is designed in such a way that each treatment occurs once and only once in each row and in each column. In biological assay there may be a particular trend, say, an increase or

decrease in the response in a single animal or in a single tissue to several consecutive doses of a drug. The Latin square design takes care of this trend. In the case of (2+2) dose assay in which two solutions of standard (S) and test (T) are compared and observations are made at two dose levels, high (H) and low (L), there are four treatments  $S_H$ ,  $S_L$ ,  $T_H$  and  $T_L$ . If each treatment is repeated four times, there are 16 observations, and the order in which each set of four treatments is given is varied in such a way that each one occupies a different position in the set in each replication. This arrangement is known as  $4 \times 4$  Latin square design.

4 × 4 Latin squares design

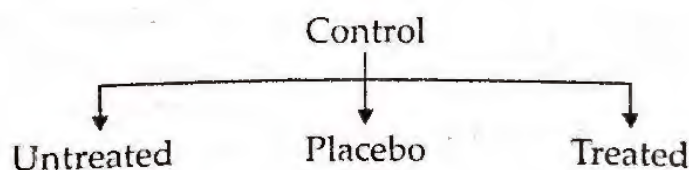
ABCD	ACBD	ADCB	ACDB
BCDA	CBDA	DCBA	CDBA
CDAB	BDAC	CBAD	DBAC
DABC	DACB	BADC	BACD

Any of the columns is selected for the experiment. Each treatment is assigned an alphabet and applied as per the arrangement in the table. By this, each treatment occurs once in each block in each order of administration. By appropriate statistical analysis it is possible to determine the variability attributable to this arrangement. The variation from this source can be subtracted from the total variation in the experiment. Thus, the degree of uncertainty in the comparison of the two solutions can thereby be decreased.

### Control

A control has to be included in every study so that some inference could be drawn about the treatment group compared to the control group. The control may be of following three types:

1. Untreated control group where no treatment is given.
2. Placebo-control group where an inert substance is given or sham operation carried out similar to that in the treated group
3. Treated-control group where a standard treatment is given or a standard technique is carried out to compare with the new treatment or technique



### Variability in the Responses

The variability in the responses arises partly from errors in measurement and partly from innate biological variations.

The error in the measurement may be of two types:

- (a) Systematic error (biased) yielding results that are consistently too high or too low and not amenable to statistical treatment.
- (b) Random error (unbiased) yielding results that vary randomly from the true value, some being larger and some smaller, and amenable to statistical treatment.

The error due to biological variation is likely to occur not only from animal to animal of the same species, but also from time to time in the same animal or in the same tissue or organ. The errors due to variability in the measurement and due to biological variation can be reduced partly by improving the technique of observation and measurement, and partly by the method of replication in a random fashion. Replication not only reduces the experimental error but actually furnishes an estimate of the error that remains. In order to obtain a valid estimate, an experiment should be self contained in respect of two things, (i) inclusion of a control group to make the experiments comparative, and (ii) estimation of the experimental error from the data of the experiment itself.

### Variation Between the Samples

Even after drawing samples by randomization there will be some chance variation between the samples. The variation may be slight or considerable. For instance, in healthy individuals variation in the body temperature is much less compared to that of the systolic blood pressure. Thus, the variation between the samples depends on the degree of variation in the population from which they are drawn. The more the members of a population included in a sample the more chance that the sample will accurately represent the population provided a random process is employed in the construction of the sample. The variations between the samples depends partly on the inherent variation in the population and partly on the size of the sample. We do not know about the variation in the population. So we use the variation in the sample (S.E.) as an estimate of it. One can usually estimate the margin of error by finding the square root of the sample size (N), then dividing 1 by that number. For instance, let us find out the margin of error of a sample of 30 observations. The square root of 30 is 5.48; by dividing 1 by 5.48 we get the value 0.18 or 18%. Therefore, the margin of error is  $\pm 18\%$ . If we increase the number of observations say, to 40, the margin of error will be  $1/\sqrt{40}$  that is 0.16. or 16%. Thus, by increasing the size of the sample we can reduce the margin of error.

## STATISTICAL ANALYSIS

### Null Hypothesis

In practice the hypothesis most often set up is that there is no difference between the two populations sampled that is the "null hypothesis" ( $h_0$ ). However, even in such a situation the sample means are most unlikely to be identical since there will be some difference between them due to chance variation. Consequently, we set

the limits within which we shall regard the samples as not having any significant difference.

### Significance

We know from the normal distribution curve that values that differ from their mean by more than twice the standard deviation are relatively rare. As a conventional level, twice the standard error is therefore adopted and a difference between the means in two samples greater than twice the standard error of the difference is said to be statistically significant at 5% level ( $p < 0.05$ ). In other words, differences of this size would occur by chance alone in five out of 100 tests or one in 20.

### Significant v. Non-Significant

A statistically non-significant result does not necessarily signify that the compound under investigation is inactive. It may indicate that the particular experiment failed to prove its effectiveness. Non-significant result may be due to a number of factors like use of wrong or insufficient subjects, or insufficient dose or concentration of the test substance. In such a situation it is better to take statistically not significant as the "non proven" of Scot's law rather than as the "not guilty" of English law.

### Sampling Error

There exist two risks in sampling that is a possibility of either of the two types of error (Fig.34.3).

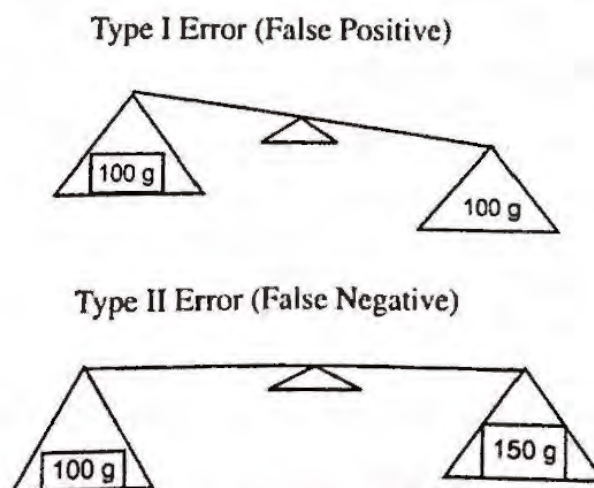


Fig. 34.3. Diagrammatic representation of the two types of errors

1. Type I Error ( $\alpha$ ) or false positive – is the probability of finding a difference when no such difference really exists.
2. Type II Error ( $\beta$ ) or false negative – is the probability of inability to detect the difference when there exists the real difference.

### Impact of Sampling Error in the Drug Screening Program

In type I error (false positive) an inactive compound is taken as an active one. However, in subsequent studies its inactivity will be revealed and thus will get rejected at a later stage.

In type II error (false negative) an active compound is taken as an inactive one and gets rejected at a very early stage and thus gets lost from the further studies.

### Ways of Reducing Sampling Errors

1. Selecting less variable population, such as the use of the inbred rather than the random bred laboratory animals.
2. Subdividing into strata of similar individuals e.g. use of the same litter, or the same sex or age, or the similar body weight etc.
3. Using sufficient number of subjects to give a reliable result.
4. Using the same animal (paired sample) or tissue for different treatments
5. Using sufficient dose or concentration of the test substance

### How Significant is Statistically Significant

The term significant has two meanings. As per the dictionary, it ordinarily means important, while statistically significant means it is probably caused by something other than mere chance. In an experiment the finding may be statistically significant but not necessarily of practical importance.

### Assumptions Made in the Significance Tests

In the test of significance of the difference between the two sample means we generally assume that the means of the two populations or their variances or both are similar. For equal sample sizes the normality and the variances of the two samples are usually equal. The test for the homogeneity of variances should be carried out if one sample is much smaller in size and has a much larger variance than the other.

### Test of Homogeneity of Variances

In order to decide whether two or more variances differ significantly we do the F test as follows:

$$F = \frac{\text{larger variance}}{\text{smaller variance}}$$

The calculated value of F is compared against the tabulated value of F at 5% probability (see Appendix). If the sample F is larger than the tabulated value; it fails in the homogeneity test. In such a situation the usual procedure is the logarithmic transformation of the data before doing the *t* test. Alternatively, a non-parametric test such as Wilcoxon's rank sum test may be employed.

### Difference Between the Two Means

In a comparative study, we usually try to find out whether the means of the



two groups differ significantly or not. We already know that that the mean of a sample has a standard error, and that a value that differs more than twice its standard error from the mean would be expected by chance only in about 5% of cases. Likewise, the difference between the two sample means (S.E.diff.) also has a standard error calculated as the sum of the standard error of the two sample means. In statistics, however, this (S.E.<sub>1</sub>+S.E.<sub>2</sub>) is calculated in a little different way, first by adding the variances of the two samples and then extracting its square root as shown below:

$$\text{S.E. diff.} = \sqrt{(\text{SD}^2/n_1 + \text{SD}^2/n_2)}.$$

### PARAMETRIC TESTS

The parametric test is performed where the data are assumed to be normally distributed. To test the null hypothesis that there is no difference between the means of the two samples, we divide the difference between the means by the standard error (S.E.) of the difference. If the difference is more than twice the S.E. it is significant and likely to occur by chance in less than 5% of cases ( $P < 0.05$ ).

#### Student's *t* Test

With smaller sample size of less than 60, the multiple of S.E. is somewhat larger than twice the S.E., and the smaller the sample size the larger it becomes. This is because of the fact that in the *t* distribution curve more values are in the extended tail ends in contrast to the normal distribution curve where there are very few values in the tail ends. Hence, in the case of small sample size some modification of the procedure of dividing the difference between the means by the S.E. is needed, and the technique thus developed is known as the Student's *t* test. This is one of the most widely used tests applied in biological investigations employing samples of small size. The *t* test has to be applied for statistical analysis when a sample consists of about 30 or less number of items. It is usually applicable to measurement (graded) data, such as blood sugar level, body weight, reaction time, etc.

It can be used under two situations:

- (i) when the comparison is made between the two different groups (between subjects comparison)
- (ii) when the comparison is made between the two measurements in the same subject following two consecutive treatments provided the first has no influence on the effect of the second (within subject comparison by the paired *t* test)

#### Difference Between the Means of the Two Samples

For a small sample size, say 30 or less, we first calculate a combined variance for the two samples in the following steps :

$SD^2 = (Sx_1^2 + Sx_2^2)/(n_1 + n_2 - 2)$ , where  $Sx_1^2$  and  $Sx_2^2$  are the respective sum of squares of deviations, and  $n_1$  and  $n_2$  the number of individuals in each sample.

Then, we calculate the standard error (S.E.) of the difference:

$$S.E.diff. = \sqrt{(SD^2/n_1 + SD^2/n_2)}$$

Finally, we get the value  $t$  by dividing the difference between the two means by the S.E. of the difference:

$$t = (\bar{x}_1 - \bar{x}_2)/S.E.diff.$$

The value thus obtained is compared against the value in table of  $t$  distribution (see Appendix) against the combined degrees of freedom ( $n_1 + n_2 - 2$ ) at the appropriate probability level.

*Example:* Testing the difference in the EC50 of acetylcholine in control and in morphine treated mouse isolated ileum (Table 34.5).

**Table 34.5**  
EC50 values of ACh ng/ml in control ( $X_1$ ) and in morphine treated ( $X_2$ ) mice.

<i>Control</i>		<i>Morphine treated</i>	
$X_1$	$X_1^2$	$X_2$	$X_2^2$
28	784	40	1600
37	1369	27	729
22	484	57	3249
30	900	26	676
31	961	60	3600
10	100	39	1521
40	1600	42	1764
33	1089	38	1444
21	441	48	2304
26	676	51	2601
10	100		
15	225		
24	576		
25	625		
$SX_1 = 352$	$SX_1^2 = 9930$	$SX_2 = 428$	$SX_2^2 = 19488$
$(SX_1)^2 = 123904$		$(SX_2)^2 = 183184$	
$\bar{x} = 25.1$		$\bar{x} = 42.8$	

<i>Parameter</i>	<i>Control</i>	<i>Treated</i>
$n$	14	10
$SX$	352	428
$\bar{x}$	25.1	42.8
$(SX)^2$	123904	183184
$SX^2$	9930	19488
$(SX)^2/n$	8850.29	18318.4
$Sx^2 = SX^2 - (SX)^2/n$	1079.71	1169.6

$$S. D.^2 = \frac{1079.71 + 1169.6}{14 + 10 - 2} = 102.24$$

$$S. E. \text{ diff} = \sqrt{\frac{102.24}{14} + \frac{102.24}{10}} = \sqrt{102.24 \left( \frac{1}{14} + \frac{1}{10} \right)} = 4.19$$

$$t = \frac{42.8 - 25.1}{4.19} = 4.22; \text{ d.f.} = 14 + 10 - 2 = 22$$

The table of *t* distribution (see Appendix) shows that at 22 degrees of freedom, the calculated *t* value 4.22 lies beyond the recorded value 3.792 at probability level 0.001, which indicates that the difference in the mean EC50 values of the two groups of mice are highly significant ( $P < 0.001$ ).

### Difference Between Means of Paired Sample

As an illustration, 10 rats were used in the study of an anorectic compound. The difference in the body weight before and after treatment was recorded in each rat as set out in Table 34.6.

Table 34.6  
Difference in the body weight of rats before and after treatment

Rat	Before treatment (A)	After treatment (B)	Difference in B.W. (A-B) or d	Squared difference d <sup>2</sup>
1	190	191	-1	1
2	208	201	7	49
3	221	219	2	4
4	188	190	-2	4
5	176	179	-3	9
6	200	196	4	16
7	210	199	11	121
8	198	191	7	49
9	205	196	9	81
10	199	187	12	144
			Sd = 46	Sd <sup>2</sup> = 478
			(Sd) <sup>2</sup> = 2116	
			$\bar{d}$ = 4.6	

$$S_x^2 = 478 - \frac{2116}{10} = 266.4$$

$$S. D. = 5.44 \quad S. E. = \frac{S. D.}{\sqrt{n}} = 1.72$$

$$S. D.^2 = \frac{S_x^2}{n - 1} = \frac{266.4}{9} = 29.6$$

$$t = \frac{\bar{d}}{S. E.} = \frac{4.6}{1.72} = 2.67$$

Consulting the table of *t* distribution (see Appendix) at 9 degrees of freedom, we find that the value 2.67 lies beyond the recorded value 2.62 at probability level 0.05. Thus, the value obtained is statistically significant ( $P < 0.05$ ).

Verification of the Published Data

An important rule in a publication is to give the reader all the information he needs so as to enable him to verify the result presented there. With the available data, such as the mean, the standard deviation (s) or the standard error.(s.e.) and the number of observations (n) we can verify the results by squaring the s.e., adding them up and finally taking the square root to get S.E.difference:

$$S.E.diff. = \sqrt{(s.e._1^2 + s.e._2^2)}$$

We get the *t* value by dividing the difference between the means by the S.E. diff.

$$t = (\bar{x}_1 - \bar{x}_2)/S.E. diff.$$

Finally, the calculated *t* value is compared with the value in the table of *t* distribution against the d.f. ( $n_1 + n_2 - 2$ ) at 0.05 probability.

As an example, we have picked up the figures from the Table 1 described by Paul, *et al.* 2004 and presented in Table 34.7

Table 34.7  
Mucosal mast cell count in stressed and in un-stressed (control) rats

	Control ( <i>x</i> )	Stress ( <i>x</i> )
Number (n)	8	8
Mean ± SE.	74.13 ± 5.04	23.38 ± 3.14
S.E. <sup>2</sup>	25.40	9.86
Sum S.E. <sup>2</sup>		35.26
S.E. diff		5.94

$$t = (\bar{x}_1 - \bar{x}_2)/S.E.diff. = (74.13 - 23.38)/5.94 = 8.54$$

Consulting the *t* table for 14 d.f. we find the calculated value 8.54 far exceeds the value 4.140 at  $P = 0.001$ . Therefore, the conclusion by the authors that the difference between control and stressed groups is highly significant ( $P < 0.001$ ) is found to be correct.

ANALYSIS OF VARIANCE (ANOVA)

While the Student's *t* test compares the means of the two samples, the ANOVA compares the variances of two or more samples. It is the most elegant, powerful and useful technique by which the total variation in the set of data is reduced to components associated with possible sources of variability. The test compares the variations (variance) between the groups with those within the groups. If 'between' and 'within' variances are approximately of the same size, there will be no significant difference between the group means. On the other hand, a comparative larger 'between' variance will indicate a significant difference between them. An assumption is made similar to *t* test that all sample groups have similar

variances. If there is some strong reason to doubt this, one has to do *a priori* test for the homogeneity of variance as already described. The one-way ANOVA test is becoming more and more popular in the scientific studies.

Steps for calculation for the one-way ANOVA or F test:

1. Enter the data (x) in respective columns (n)
2. The data in rows indicate replicates (k)
3. Add up the values in each column ( $x_1 + \dots + x_k$ ) and obtain the sum  $Sx_1, Sx_2, \dots, Sx_n$  against each column
4. Add up the column totals ( $Sx_1 + \dots + Sx_n$ ) to get the grand total of all items (SX)
5. Square up the grand total to get  $(SX)^2$
6. Divide each  $Sx$  by the number of replicates  $k$  to get the mean of each column ( $\bar{x}$ )
7. Square up each item in a column and add up to get sum of squares ( $Sx^2$ ) for each column
8. Add up all the  $Sx^2$  to get  $SX^2$  (A)
9. Divide  $(SX)^2$  by  $N$  the total number of items to get the 'correction factor' (C)
10. Square up each column total and divide by the number of replicates  $k$  to get  $(Sx)^2/k$  for each column total and finally add them up (B)

**Computation:**

Total number of items  $N =$  Number of groups (n) multiplied by number of replicates (k)

$$= nk$$

Total sum of squares = A - C

Between groups (treatments) sum of squares = B - C

Within group (error) sum of squares = A - B

Degrees of freedom (d.f.): Total number of items =  $N - 1$

Number of treatments =  $n - 1$

Error =  $N - n$

By dividing the sum of squares by the respective d.f. we get the mean square. Finally, we divide the between groups mean square by the within groups mean square to get the value for F (Table 34.8).

Table 34.8  
Analysis of Variance

Sources of variation	d.f.	Sum of squares	Mean square	F
Between groups	$n - 1$	$SS_{tr}$	$SS_{tr}/d.f. = MS_{tr}$	$MS_{tr} / MS_{er}$
Within groups (Error)	$N - n$	$SS_{er}$	$SS_{er}/d.f. = MS_{er}$	
Total	$N - 1$	$SS_{ta}$		

*Example:* Three groups of six rats each were assigned three treatments, that is control, tea root extract and acetylsalicylic acid to compare their effect on the weight

of the cotton pellet granuloma as presented in the Table 34.9. The aim of this study was to find out whether there was any significant difference between the three groups of treatment. For this we performed the one-way ANOVA or F test named after R A. Fisher, the renowned statistician.

Table 34.9  
Increase in the weight (g) of the cotton pellet granuloma in control and in treated groups\*

	Control ( $x_1$ )	Tea root ext. ( $x_2$ )	Acetylsalicylic acid ( $x_3$ )	
	0.050	0.040	0.065	
	0.125	0.030	0.055	
	0.085	0.045	0.050	
	0.120	0.055	0.070	
	0.105	0.050	0.060	
	0.145	0.055	0.065	
Sx	0.63	0.275	0.365	SX = 1.27
Mean ( $\bar{x}$ )	0.105	0.046	0.061	
Sr <sup>2</sup>	0.072	0.013	0.022	SX <sup>2</sup> = 0.107 (A)
(Sx) <sup>2</sup> / k	0.066	0.013	0.022	= 0.101 (B)

\* Raw data by courtesy of Dr. J.R.Vedasiromoni

Computation:

Total number of observations  $N = n.k = 18$

Correction factor =  $(SX)^2 / N = (1.27)^2 / 18 = 0.090$  (C)

Total sum of squares =  $(A - C) = 0.107 - 0.090 = 0.017$  (TSS)

Between group sum of squares =  $(B - C) = 0.101 - 0.090 = 0.011$  (BSS)

Within group sum of squares (error)  $(TSS - BSS) = 0.017 - 0.011 = 0.006$

Degrees of freedom (d.f.): Total number of observations  $(N - 1) = 17$

Number of groups (treatments) =  $(n - 1) = 2$

Error =  $17 - 2 = 15$

By dividing the sum of squares by the respective d.f. we get the mean squares (variances). Finally, we divide the 'between group mean square' by the 'within group mean square' to get the value of F (Table 34.10)

Table 34.10  
Analysis of Variance

Sources of variation	d.f.	Sum of squares	Mean squares	F
Between treatments	2	0.011	0.006	15
Within treatments (Error)	15	0.006	0.0004	
Total	17	0.017		

Referring to the table of distribution of F (see Appendix) against 2 d.f. for greater mean square and 15 d.f. for lesser mean square, we find a value 6.36 at 1% level of

significance. Since the experimental value of 15 is far greater than the recorded value we conclude that the differences between the groups are highly significant ( $P < 0.01$ ).

Like the  $t$  test the analysis of variance (F test) can also be applied in experiments involving only two sample groups. The F test is relatively easier to perform than the  $t$  test. It is, however, up to the investigator to select the test of his choice.

### DUNNETT'S TEST

A significant F test tells us that there are differences between the groups as a whole and not which group differs from the other. In case we want to know specifically which group differs from the other, we may have to take recourse to a *post hoc* analysis by a specialized test known as the Dunnett's test named after the statistician who developed it. It is conducted by computing a modified form of  $t$  test between the different groups using the formula:

$t_d = (M_i - M_c) / \sqrt{(2MSE/HM)}$ , where  $M_i$  is the mean of the  $i$ th experimental group,  $M_c$  is the mean of the control group, MSE is the mean square error as computed from the analysis of variance, HM is the harmonic mean of the sample sizes of all the experimental groups including control.

$HM = n / (1/k_1 + 1/k_2 + \dots + 1/k_n)$  where  $n$  is the number of sample groups and  $k$  the number of items in each sample.

The degrees of freedom (df) for the test are equal to  $N - n$ , where  $N$  is the total number of subjects in all groups and  $n$  is the number of groups including the control.

*Example*: Having found the F test significant in the earlier example, we wanted to know specifically whether there is significant difference between the tea root extract group (Mean 0.046) and the control group (Mean 0.105). We carried out a *post hoc* analysis by the Dunnett's test as follows:

Comparing the two groups, we first work out the harmonic mean (HM) and then the  $t_d$ :

$$HM = 3 / (1/6 + 1/6 + 1/6) = 5.90$$

$$t_d = (0.105 - 0.046) / \sqrt{(2 \times 0.0004 / 5.90)} = 0.059 / 0.012 = 4.92$$

The degrees of freedom  $(18 - 3) = 15$

Consulting the Dunnett table (see Appendix) at 15 d.f. we find the value 3.25 against group 3 at 0.01 probability. The calculated value 4.92 is larger than the stated value 3.25 indicating the difference between the tea root extract group and the control group is highly significant ( $P < 0.01$ ).

Now, we want to know whether there is any significant difference between the tea root extract group and the acetylsalicylic acid group. We calculate the  $t_d$  value as follows:

$$t_d = (0.061 - 0.046) / \sqrt{(2 \times 0.0004 / 5.90)} = 0.015 / 0.012 = 1.25$$

Consulting the Dunnett Table against 15 d.f. we find the value 2.44 against group 3 at 0.05 probability is much higher than the calculated value 1.25 indicating that there is no significant difference between the two treatment groups ( $P > 0.05$ ).

NON-PARAMETRIC TESTS

The non-parametric or distribution-free methods are becoming increasingly popular in the analysis of biological data because of the following:

- (a) It is simpler to use without much mathematical calculations, and
- (b) Unlike in the *t* test, no assumption is made regarding the nature of the distribution so that the test can be applied irrespective of the data being normally distributed.

Wilcoxon's Signed Rank Test (Paired Data)

We take the experiment described earlier where the differences in the body weight in rats before and after an anorectic compound have been recorded. The results are arranged in a tabular form (Table 34.11) in the following steps :

1. Rank the differences in the body weight in an increasing order irrespective of the signs, the smallest value being given rank 1.
2. Average ranks are given for the tied values.
3. Zero differences, if any, are excluded and the sample size reduced accordingly.
4. The rank total must correspond to the value obtained by the formula :  $n(n+1)/2$ , where *n* is the number of items (rats).
5. The respective sign is attached to each rank.
6. The plus ranks and the minus ranks are added separately, and the smaller total is taken for the test of significance.
7. Irrespective of its sign, it is referred to the table of Wilcoxon test on paired samples against the number of pairs in the sample (see Appendix).
8. Larger rank totals than those recorded in the table are non-significant at the level of the probability shown.

Table 34.11  
Ranking of the differences in the body weight in each rat before and after treatment

Rat	Differences in B.W.(g)	Rank	Rank Average	Signed Rank
1	1	1	1	1
2	-2	2	2.5	-2.5
3	2	3	2.5	2.5
4	3	4	4	4
5	-4	5	5	-5
6	-7	6	6.5	-6.5
7	-7	7	6.5	-6.5
8	-9	8	8	-8
9	-11	9	9	-9
10	-12	10	10	-10

In the present example, the rank total adds up to 55, the plus ranks = 7.5, the minus ranks = 47.5. Number of paired observations = 10. Consulting Wilcoxon table of paired observations, the smaller value 7.5 is less than the recorded value 8



at 5% level against 10 pairs. Hence, the result obtained is significant at 5% level ( $P < 0.05$ ).

### Wilcoxon's Two-Sample Rank Test (Unpaired Data)

This test is applied to unpaired samples that need not be of equal size. Steps for calculation are as follows:

1. The observations in two samples (groups) are combined into a single series and ranked in order of magnitude; the figures from one sample are distinguished from those of the other, say, by underlining.
2. Average ranks are given for the tied values; the total should tally with the calculated value by the formula  $n(n+1)/2$ , where  $n$  is equal to the total of both the samples.
3. The ranks of any one sample are added; if the total ( $T_1$ ) is more than half the rank total of the two samples together, the other rank total ( $T_2$ ) is found out by the formula  $T_2 = [n(n+1)/2] - T_1$ .
4. The smaller of the two rank totals is referred to the table of Wilcoxon test on unpaired samples (see Appendix) with  $n_1$  = number of observations in smaller sample, if any, and  $n_2$  = number of observations in the other sample. If the calculated total ( $T_1$  or  $T_2$ ) is equal or less than the tabulated value, we reject the null hypothesis at the corresponding significance level.

*Example:* The data in Table 34.12 represent the number of jumps per mouse observed during naloxone precipitated morphine withdrawal in control and in atropine treated mice.

Table 34.12  
Number of jumps per mouse in the control and in the treated group

Control Mice	No. of jumps	Treated Mice	No. of jumps
1	5	1	15
2	2	2	9
3	13	3	11
4	6	4	5
5	5	5	31
6	8	6	36
7	2	7	9
8	5	8	18
9	9	9	23
10	17	10	21

The values (number of jumps) of two groups are combined together and ranked in an increasing order (underlined figures indicate values from treated mice) presented in Table 34.13. Average ranks are entered for the same values.

**Table 34.13**  
Ranking of number of jumps in the control and in the treated group combined together

No. of jumps	Ranks	Average ranks	No. of jumps	Ranks	Average ranks
2	1	1.5	9	11	10
2	2	1.5	11	12	12
5	3	4.5	13	13	13
5	4	4.5	15	14	14
5	5	4.5	17	15	15
5	6	4.5	18	16	16
6	7	7	21	17	17
8	8	8	23	18	18
9	9	10	31	19	19
9	10	10	36	20	20

The average rank total is calculated by adding up the individual average ranks. This can also be calculated by the formula  $n(n+1)/2$ , where  $n$  is total number of ranks.

In the present case the average rank total =  $20(20 + 1)/2 = 210$

Control = 69.5 Treated =  $210 - 69.5 = 140.5$

With 10 mice in each group ( $n_1 = n_2 = 10$ ) the smaller value 69.5 is less than the recorded value 71 in the table of Wilcoxon test on unpaired samples at 1% level of probability. Hence, the difference is highly significant ( $P < 0.01$ ).

**CORRELATION**

Correlation is the mutual relationship between the two measurements. There are usually two variables, the independent variables that are predetermined, and the dependent variables that vary proportionately with changes in the independent variables. With the progressive increase in the size of the independent variable there may be either an increase (positive correlation) or a decrease (negative correlation) in that of the dependent variable. The correlation between the two variables does not necessarily mean a cause and effect relationship but simply an association between the two.

**Correlation Coefficient (r)**

It is a measure of covariation, the degree of association between the two variables. The independent variable is plotted along the horizontal axis (abscissa), while the dependent variable is plotted along the vertical scale (ordinate). In biological science, the values plotted in the form of a scatter diagram generally occupy an elliptical area, the overall impression of which may be approximately of a linear nature (linear regression). The correlation coefficient is measured on a scale ranging from +1 to -1. Complete correlation between the two variables in the shape of a straight line is expressed by +1 when the dependent variable increases with the increase in the independent variable, and by -1 when the dependent variable decreases with the increase in the independent variable. When the

individual plots are scattered in an elliptical shape instead of in a perfect straight line the correlation coefficient is less than 1. Complete absence of correlation where the individual plots are scattered almost in the shape of a circle is expressed as 0. *Calculation of the correlation coefficient.* The formula for the calculation of the correlation coefficient ( $r$ ) is as follows :

$$r = S_{xy} / \sqrt{(S_x^2 \times S_y^2)}, \text{ where } \begin{aligned} S_{xy} &= SXY - [(SX)(SY)]/N \\ S_x^2 &= SX^2 - (SX)^2/N \\ S_y^2 &= SY^2 - (SY)^2/N \end{aligned}$$

*Example:* The weight gain following high protein diet in eight rats are presented for the calculation of the correlation coefficient (Table 34.14).

Table 34.14  
Weight gain in rats following high protein diet

Rat	Initial weight (X)	Gain in weight (Y)	X <sup>2</sup>	Y <sup>2</sup>	XY
1	60	112	3600	12544	6720
2	48	118	2304	13924	5664
3	64	159	4096	25281	10176
4	46	82	2116	6724	3772
5	50	128	2500	16384	6400
6	57	107	3249	11449	6099
7	45	103	2025	10609	4635
8	74	133	547	17689	9842
N = 8	SX = 444	SY = 942	SX <sup>2</sup> = 25366	SY <sup>2</sup> = 114604	SXY = 53308
$\bar{x}$ = 55.50	$\bar{y}$ = 117.75	(SX) <sup>2</sup> = 197136	(SY) <sup>2</sup> = 887364		

**Computation:**

$$S_{xy} = SXY - (SX)(SY)/N = 53308 - [(444 \times 942)/8] = 1027$$

$$S_x^2 = SX^2 - (SX)^2/N = 25366 - (197136/8) = 724$$

$$S_y^2 = SY^2 - (SY)^2/N = 114604 - (887364/8) = 3684$$

$$r = S_{xy} / \sqrt{(S_x^2 \times S_y^2)} = 1027 / \sqrt{(724 \times 3684)} = 0.63$$

The correlation coefficient ( $r$ ) thus works out to be 0.63.

**Standard Error of  $r$  :**

To test the deviation of  $r$  from 0, or nil correlation, we use the  $t$  test as follows:

$$t = r \times \sqrt{[(N - 2)/(1 - r^2)]}; \text{ d.f.} = N - 2$$

In the above example, the number of rats  $N = 8$ . Applying the above formula, we have:

$$t = 0.63 \times \sqrt{[(8-2)/(1-0.63^2)]} = 0.63 \times \sqrt{(6/0.6)} \\ = 0.63 \times 3.16 = 1.99$$

Consulting the  $t$  table at 6 d.f., we find the value 1.99 is less than the recorded value 2.447 at probability 0.05. The correlation coefficient may thus be regarded as not significant ( $P > 0.05$ ).

### REGRESSION

In regression the average value of  $y$  (dependent variable) changes with  $x$  (independent variable). This relationship between  $y$  and  $x$  is represented by a simple equation known as the regression equation. When it changes approximately in a linear fashion it is known as the linear regression.

#### Regression Equation

The regression equation is represented by the following formula:

$$Y = a + bX$$

where  $Y$  is the dependent variable,  $a$  is the point on the ordinate (vertical axis) at which the regression line meets,  $b$  (regression coefficient) the slope of the regression line and  $X$  the independent variable.

#### Calculation of Regression Equation

$$b = S_{xy}/S_x^2$$

$$a = \bar{y} - b\bar{x}$$

Applying the data in Table 34.14 for correlation coefficient, we get the following:

$$b = 1027 / 724 = 1.42$$

$$a = 117.75 - (1.42 \times 55.50) = 38.94$$

Therefore, the regression equation of the values in Table 34.14 is as follows:

$$Y = a + bX = 38.94 + 1.42 X$$

This means that on average, for each gram of increase in initial weight there is 1.42 grams of increase in gain over the range of measurements made.

In order to draw the regression line as per the equation, we take three values of  $X$ , one a lower, one a middle and one a higher value, and substitute these in the equation as follows:

$$\text{If } X = 48, Y = 38.94 + (1.42 \times 48) = 107$$

$$\text{If } X = 57, Y = 38.94 + (1.42 \times 57) = 120$$

$$\text{If } X = 64, Y = 38.94 + (1.42 \times 64) = 130$$

We plot these three points and then draw a line through them as shown in the Fig. 34.4

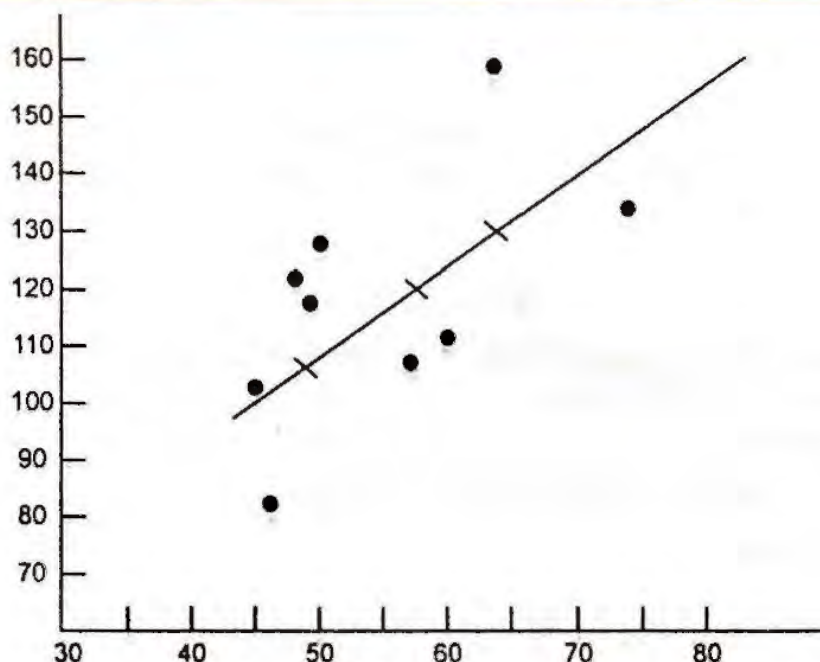


Fig. 34.4 Scatter diagram. Abscissa: Initial body weight (g.). Ordinate: gain in body weight. Black dots: original values. Lines: calculated values as per the regression equation.

In order to test the statistical significance of the regression coefficient, we compute the value of  $t$  as follows:

$$t = b/s_{b.}, \text{ where } s_{b.} = \sqrt{(S_{yx^2}/S_x^2)};$$

$$S_{yx^2} = Sd_{yx^2}/(N - 2);$$

$$Sd_{yx^2} = S_y^2 - (S_{xy})^2/S_x^2$$

$$\text{Thus, } Sd_{yx^2} = 3684 - [(1027)^2/724] = 3684 - 1457 = 2227$$

$$S_{yx^2} = 2227/6 = 371$$

$$s_{b.} = \sqrt{(371/724)} = 0.71$$

Therefore,

$$t = 1.42/0.71 = 2.0$$

Consulting the table of  $t$  distribution, we find the value 2.0 is less than the value recorded against 6 d.f. (2.447) at 0.05 probability indicating that the regression coefficient ( $b=1.42$ ) is not statistically significant ( $P > 0.05$ ).

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# Appendix

## GUIDE TO DRUG DOSES IN LABORATORY ANIMALS

<i>Drug</i>	<i>Species</i>	<i>Dose per kg body weight and route</i>	<i>Remarks</i>
Acetylcholine Chloride	Dog	1 to 10 $\mu\text{g}$ iv 0.1 mg iv	Muscarinic (nicotinic in high dose) agonist
	Cat	5 to 10 $\mu\text{g}$ iv	
	Rabbit	10 $\mu\text{g}$ iv	
	Rat	1 to 10 $\mu\text{g}$ iv	
Adrenaline bitartrate	Dog	0.5 to 3 $\mu\text{g}$ iv	Adrenoceptor agonist
	Cat	1 to 5 $\mu\text{g}$ iv	
	Rabbit, Guinea pig & Rat	3 $\mu\text{g}$ iv	
Alloxan monohydrate	Dog	50 to 75 mg iv	Produces experimental diabetes mellitus 1 week before experiment 24 hours before experiment 3 days before experiment
	Rabbit	150 to 200 mg iv	
	Rat	50 mg iv 150 mg po	
	Mouse	80 mg iv	
Alvimopan	Rat	0.1 to 3 mg/kg po 1 mg/kg iv	Selective opioid $\mu$ receptor antagonist
Amantadine HCl	Rat	10 to 30 mg ip	DA uptake blocker
Aminoguanidine HCl	Mouse	100 mg po	NOS* inhibitor
Aminoxyacetic acid	Rat	20 mg ip	Increases central GABA level; 60 min prior to test
Amitriptyline HCl	Mouse	10 mg ip	DA uptake blocker
d-Amphetamine sulphate	Rat	3 to 5 mg sc 0.7 mg sc	CNS stimulant and anorectic; increases locomotor activity
Angiotensin II amide	Dog	0.4 $\mu\text{g}$ iv	Produces hypertension
	Cat	0.02 to 0.1 $\mu\text{g}$ iv	
Apomorphine HCl	Dog	0.1 mg sc	Emetic Dopaminergic agonist
	Guinea pig	2 to 8 mg sc	
	Rat	0.2 to 0.8 mg sc	

\* Nitric oxide synthases

<i>Drug</i>	<i>Species</i>	<i>Dose per kg body weight and route</i>	<i>Remarks</i>
Aspirin	Mouse	0.1 to 1.0 mg ip	To induce gastric ulcers
	Rat	200 mg po	
Atenolol	Dog	0.3 mg iv	$\beta_1$ -Adrenoceptor blocker; 5 min prior to test
Atropine methylnitrate	Rabbit	0.5 mg iv	Peripheral muscarinic receptor blocker
	Rat	2.5 to 5 mg sc	
	Mouse	2 to 5 mg ip	
Atropine sulphate	Dog	0.5 to 1 mg iv	Muscarinic receptor blocker
	Rabbit	2 mg iv	
	Rat	1 mg iv	
	Mouse	2 to 10 mg ip	
Baclofen HCl	Mouse	1 mg ip	GABA antagonist
Benserazide HCl	Guinea pig	50 mg ip x 14 days	Peripheral DOPA decarboxylase inhibitor
	Rat	50 mg ip 3 times at 2 hourly intervals	In lower doses only peripheral depletion
		200 mg ip 3 times at 2 hourly intervals	Both central and peripheral noradrenaline (NA) depletion in high doses
Mouse	50 mg ip	30 min before experiment	
Benztropine	Rat	1 to 6 mg ip	DA uptake blocker
Bretylium tosylate	Rat	5 to 20 mg iv	Adrenergic neuron blocker; 2 h prior to test
Bromocriptine mesylate	Rat	1 to 5 mg sc	Dopaminergic agonist
Butaclamol	Rat	5 to 10 mg po	Dopaminergic antagonist
Candesartan	Rat	5 to 10 $\mu$ g iv	AT <sub>1</sub> -blocker
Carbachol chloride	Rat	0.2 to 0.5 $\mu$ g iv	Gastric acid stimulation
Carbon tetrachloride	Rat	0.1 to 1 ml po	Induction of liver damage total dose
Carrageenan	Rat	0.1 ml of 10% soln.	Phlogistin subplantar
DL-p Chloroamphetamine HCl (PCA)	Mouse	5 mg ip	5HT depletor; 24 h before experiment



<i>Drug</i>	<i>Species</i>	<i>Dose per kg body weight and route</i>	<i>Remarks</i>
p-Chlorophenylalanine (PCPA)	Rabbit	125 mg daily for 3 days ip	5-HT depletor; depletes 5-HT to the maximum on 4th day; results in 80% depletion of 5-HT and much less of NA and dopamine (DA)
	Rat	300 mg daily for 3 days ip	
	Mouse	100 mg every 48 h for 5 days sc 150 to 300 mg 12 hourly for 3 days ip	
DL-p-Chlorophenylalanine methylester HCl	Rat	100 mg daily for 4 days ip 150 to 300 mg daily for 3 days ip	Soluble form of PCPA to be stored at 0°C; experiment to be done 12 h after the last dose
Cimetidine	Rat	100 mg ip	H <sub>2</sub> -Receptor blocker
Cinanserin HCl	Rat	10 mg ip	5-HT blocker; 30 min prior to test
	Mouse	5 mg ip	
Ciproxifan	Rat	0.3 to 3 mg po	H <sub>3</sub> -antagonist
Clobenpropit dihydrobromide	Rat	3 to 30 mg po	H <sub>3</sub> -antagonist
Clomipramine HCl	Rat	10 mg ip	NA uptake blocker
Cocaine HCl	Dog	5 mg iv	Neuronal catecholamine (CA) uptake blocker
	Cat	2 mg ip 6 mg im	
	Mouse	4 mg ip	
Cromoglycate or cromolyn sodium	Rat	2.5 to 5 mg × 2 daily for 4 days po	Mast cell stabilizer
Cyproheptadine HCl	Dog	40 to 100 µg iv	5-HT and histamine blocker
	Guinea pig	250 µg ip	
	Rat	50 µg sc	
Decamethonium bromide	Dog	0.2 mg iv	Depolarizing neuromuscular blocker
	Cat	15 µg iv	
	Rabbit	1 mg iv	
Deoxycorticosterone acetate (DOCA)	Rat	25 mg × 2 tab sc implantation 5 mg sc twice a week × 7 weeks	Produces hypertension in unilateral nephrectomized rat Drinking water replaced by 0.9% NaCl after 5 to 6 weeks

<i>Drug</i>	<i>Species</i>	<i>Dose per kg body weight and route</i>	<i>Remarks</i>
Desmethylimipramine (Desipramine)	Rat	0.1 to 0.5 mg iv	Neuronal uptake inhibitor of CA 1 h before experiment
	Mouse	25 mg ip 20 mg ip	
Diethyldithiocarbamate sodium (DDC)	Rabbit	400 mg ip	Brain NA depletion by 46% 6 h after sc administration; DA is increased and 5-HT remains unchanged
	Mouse	500 mg ip followed after 2 h by 500 mg sc	
Diethylstilboestrol (Stilboestrol)	Rat	1 mg ip/im 0.1 mg im/sc	24 h prior to experiment for priming young female rats
Digoxin	Dog	0.2 mg iv	To induce cardiac arrhythmia
	Cat	0.15 mg iv	
Dihydroergotamine tartrate	Dog	10 mg iv	$\alpha$ -Adrenergic blocker
	Rabbit	1 mg iv	
	Rat	0.5 mg iv	
1,1-Dimethyl-4-phenyl- piperazinium iodide (DMPP)	Dog	0.15 mg iv	Ganglionic stimulant
	Cat	0.2 mg iv	
Diphenhydramine HCl	Dog	2 mg iv	$H_1$ -Receptor blocker
	Cat	2 mg iv	
	Guinea pig	8 mg ip	
Dipyridamole	Cat	1 mg iv	Phosphodiesterase inhibitor
	Rat	10 mg iv	
Disulfiram (Tetraethylthiuram disulfide)	Rabbit & Rat	100 mg ip	NA synthesis inhibitor (dopamine- $\beta$ -oxidase and aldehyde dehydrogenase inhibitor)
Domperidone HCl	Rat	0.1 to 25 mg ip 6 mg sc	DA ( $D_2$ ) antagonist
L-DOPA HCl	Guinea pig	10 mg ip	Dopaminergic agonist (to alter motor activity)
	Rat	2.5 mg ip	
	Mouse	2 to 6 mg sc	
Dopamine	Rat	1 to 10 mg sc	Dopaminergic agonist (to alter motor activity)
	Mouse	4 to 12 mg sc	
Egg albumin or ovalbumin	Guinea pig	A total dose of 1 ml sc	Sensitization for anaphylactic and 1 ml ip of a 100% reaction

<i>Drug</i>	<i>Species</i>	<i>Dose per kg body weight and route</i>	<i>Remarks</i>
Epibatidine dihydrochloride	Rat	1 to 3 $\mu$ g sc	suspension of crystalline egg albumin in 0.9% saline 3 weeks before experiment N* agonist
Ergotamine tartrate	Dog Cat Rabbit	8 mg iv 1.0 mg iv 0.15 mg iv	$\alpha_1$ -Adrenergic blocker
Eserine salicylate	Dog & Cat Rat Mouse	0.05 to 0.5 mg iv 0.25 mg ip 0.1 mg sc	Anticholinesterase; 20 min before experiment
Evans blue	Rat	20 to 40 mg iv	1 to 2% aq. soln. injected 5 min before experiment
Femoxetine	Rat	2.5 to 10 mg ip	5-HT neuronal uptake inhibitor
Fenfluramine HCl	Rat	15 mg po 5 to 10 mg sc/ip	Anorexigenic agent; 5-HT depletor; depletes 5-HT by 50%; releases NA by 40%, DA by 3% 24 h after administration
Fenoldopam	Rat	10 mg po	Dopaminergic agonist
FLA-63	Rat Mouse	25 to 50 mg ip 40 to 50 mg ip	NA synthesis blocker (dopamine- $\beta$ -hydroxylase inhibitor) without affecting DA and 5-HT stores; action within 2 to 5 h
Flumazenil	Cat Mouse	5 $\mu$ g iv 10 to 30 mg ip	GABA <sub>A</sub> antagonist
Fluoxetine experiment	Rat	75 to 100 $\mu$ g  3 mg po	5-HT uptake inhibitor (central total icv effect); 30 min before
Fluvoxamine maleate	Rat	10 to 20 mg ip	5-HT uptake blocker
Formalin	Rat	0.1 mg of 2% soln. under plantar aponeurosis	Phlogistogen ; repeated after 48 h

\* Nicotinic

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<i>Drug</i>	<i>Species</i>	<i>Dose per kg body weight and route</i>	<i>Remarks</i>
Gallamine	Dog	0.4 mg iv	Competitive neuromuscular blocker
	Cat	1 to 5 mg iv	
	Rabbit	0.6 mg iv	
Guanethidine sulphate	Dog	10 to 15 mg iv	Adrenergic neuron blocker; 6 h before experiment
	Cat	15 mg iv	
	Rabbit	3 mg iv	
	Guinea pig	15 mg sc	
	Rat	1 to 5 mg iv	
HA-966	Rat	10 to 40 mg iv	DA blocker
Haloperidol	Rat	0.5 to 5 mg ip	DA antagonist
		10 mg sc	
	Mouse	0.5 to 2.5 mg ip 10 mg sc	
Hemicholinium-3	Rat	5 mg ip	ACh synthesis blocker
Heparin sodium	Dog	8 mg iv	Anticoagulant
	Cat	10 mg iv	
	Rabbit	30 mg iv	
	Rat	20 mg iv	
Hexamethonium bromide	Dog	10 mg iv	Ganglion blocker
	Cat	0.5 to 5 mg iv	
	Rabbit	10 mg iv	
	Guinea pig	5 mg iv	
	Rat	10 to 20 mg iv	
Histamine dihydrochloride or acid phosphate	Dog	0.2 to 5 µg iv	Gastric acid stimulant
	Cat	0.1 to 5 µg iv	
	Rabbit	30 to 150 µg iv	Bronchospasm
	Guinea pig	0.1 mg iv	
	Rat	0.1 to 0.7% aerosol 0.25 to 1 mg iv total dose	
Hyaluronidase	Rat	A total dose of 0.4 ml of 600 IU/ml in saline sc on the dorsum of the foot	For testing anti-inflammatory agents
Hydrocortisone	Rat	10 to 40 mg sc	Anti-inflammatory agent
6-Hydroxydopamine (6 OHDA) HBr	Guinea pig	30 mg ip	Neurotoxic to NA and DA nerve terminals; administered 36 h before experiment

<i>Drug</i>	<i>Species</i>	<i>Dose per kg body weight and route</i>	<i>Remarks</i>
		50 to 200 mg ip	Administer for 2 consecutive days and carry out the experiment on the third day
	Rat	250 µg icv in 25 µl 50 mg iv (2 doses of this within 24 h followed a week later by 2 × 100 mg dose)	Experiment to be performed 24 h after but within 1 week after the last dose
		150 mg × 14 days sc 50 mg × 2 ip on day 1 and 100 mg × 2 ip from day 2 to 4	Start experiment on day 5 or day 6
6-Hydroxydopamine (6-OHDA) HCl	Cat	20 mg iv	Start experiment after 3 days
	Guinea pig	2 × 25 mg iv on day 1; 2 × 50 mg iv from day 2 to day 6	Start experiment between day 8 to 10 days
	Mouse (newborn)	50 mg × 2 doses followed 8 days later by a single dose of 100 mg iv	24 h after the 3rd dose the animal is killed
5-Hydroxytryptamine (Serotonin) creatinine sulphate	Dog	25 µg iv	For cardiovascular effects
	Guinea pig	2% (base) aerosol	To produce bronchospasm
5-Hydroxytryptophan (5-HTP) methylester	Rat	2 to 25 mg ip	Precursor of 5-HT; tested 30 min after administration
	Mouse	25 to 50 mg ip	
Hyoscine HBr	Dog	1 mg sc	Muscarinic blocker; 15 min before experiment
	Rabbit	5 mg iv	
	Rat	0.05 to 1 mg sc	
	Mouse	0.5 to 4 mg sc	
Imetit	Mouse	50 mg ip	H <sub>3</sub> -agonist
Imipramine HCl	Rat	20 mg ip	Neuronal uptake blocker of CA
	Mouse	50 to 100 mg ip	
Immepip	Rat	5 mg sc	H <sub>3</sub> -agonist
Indomethacin	Rat	4 to 5 mg ip	Anti-inflammatory agent
Iproniazid phosphate	Dog	65 mg iv	MAO inhibitor
	Rabbit	100 mg iv	
	Rat	50 mg ip	
Isoprenaline HCl	Dog	2 to 5 µg iv	β-Adrenoceptor agonist
	Cat	0.1 to 5 µg iv	
	Rabbit	2 µg iv	
	Guinea pig	10 µg iv	

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<i>Drug</i>	<i>Species</i>	<i>Dose per kg body weight and route</i>	<i>Remarks</i>
Kainic acid	Rat	5 to 500 $\mu$ g sc	24 h before experiment to induce myocardial necrosis
	Rat	1 to 10 $\mu$ g icv	Neurotoxic to GABAergic neurons
Leptazol	Rabbit	60 mg iv	Convulsant
	Rat	70 to 112 mg ip/sc	
	Mouse	0.2 ml of 6% soln. total iv	
Lithium chloride	Rat	150 mg ip	Produces behavioral changes (head twitches)
	Mouse	40 to 300 mg ip	
Mecamylamine HCl	Rat	3 mg sc	N-antagonist
Mephenesin	Dog	5 to 25 mg iv	Strychnine antagonism and inhibition of polysynaptic reflexes
	Rat	80 mg im	
	Frog	50 mg sc	
Mepyramine maleate	Dog	5 mg iv	$H_1$ -Receptor blocker
	Cat	10 mg iv	
	Guinea pig	2 mg sc	
	Rat	5 to 10 mg ip	
	Mouse	2.5 to 25 mg ip	
Metaraminol bitartrate	Rat	5 mg ip	NA uptake blocker
Metergoline	Rat	0.5 mg ip	5-HT antagonist; 1 to 2 h prior to experiment
Methacholine chloride	Dog	2 $\mu$ g iv	Gastric acid stimulant
	Rabbit	2 $\mu$ g iv	
	Rat	5 to 10 $\mu$ g iv total dose	
$\alpha$ -Methyldopa hydrazine	Rabbit	25 mg ip	Dopa decarboxylase inhibitor; depletion of peripheral NA stores
	Rat	200 mg im	
$\alpha$ -Methyl histamine dihydrochloride	Rat	100 mg po	$H_2$ agonist
$\alpha$ -Methylparatyrosine HCl	Rabbit	80 mg 4 times 12 hourly ip	Tyrosin hydroxylase inhibitor producing NA and DA depletion; maximal depletion of CA occurs 12 h after adminis-
	Rat	150 mg iv 200 mg ip	

<i>Drug</i>	<i>Species</i>	<i>Dose per kg body weight and route</i>	<i>Remarks</i>
$\alpha$ -Methylparatyrosine methylester HCl	Mouse	100 mg sc 30 to 50 mg icv	tration; administered 16 to 18 h before experiment  CA synthesis blocker (water soluble to be stored at 0° C); depletion occurs maximum by 4 h
	Rat	100 to 200 mg ip	
	Mouse	150 to 250 mg ip 100 to 250 mg ip	
Methysergide bimaleate	Rat	20 mg ip	5-HT antagonist; 30 min before experiment
	Mouse	1 to 5 mg iv	
Metiamide	Rat	25 to 100 mg ip	H <sub>2</sub> -receptor blocker; 30 min prior to experiment
	Mouse	25 to 200 mg total icv	
Metoclopramide dihydrochloride	Guinea pig	78 mg sc	Selective dopamine (D <sub>2</sub> ) antagonist; to induce catalepsy or stereotyped behaviour
	Rat	10 to 50 mg ip	
	Mouse	3 mg im	
Metoprolol tartrate	Rat	0.2 to 5 mg sc	Selective $\beta_1$ -adrenoceptor blocker
Mianserin HCl	Rat	13 mg ip	5-HT blocker; 30 min prior to experiment
Morphine sulphate or HCl	Cat	5 to 10 mg ip	Analgesic
	Guinea pig	10 mg sc	
	Rat	1.5 to 10 mg sc	
	Mouse	1 to 5 mg im	
Muscimol	Rat	10 to 30 mg ip 0.05 to 0.2 mg total icv	GABA agonist; 30 min prior to test
	Mouse	3 mg ip	
N-0437	Rat	0.5 to 2 mg ip	Dopaminergic agonist
Nadolol	Dog	0.1 mg iv	Selective $\beta_1$ -adrenoceptor blocker
Nalorphine HCl	Dog	2 mg iv	Opioid antagonist with partial agonist activity
	Cat	3 mg iv	
	Rabbit	5 mg iv	
	Rat	5 mg ip	
	Mouse	1 to 5 mg ip	
Naloxone HCl	Dog & Cat	1 to 5 mg sc	Opiate antagonist
	Rat & Mouse	0.1 to 1 mg sc	
	Mouse	1 to 5 mg ip	Precipitation of morphine withdrawal
	Mouse	1 to 5 mg ip	

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<i>Drug</i>	<i>Species</i>	<i>Dose per kg body weight and route</i>	<i>Remarks</i>
Neostigmine bromide	Dog	0.17 mg iv	Cholinesterase inhibitor
	Cat	25 to 75 µg iv	
	Rabbit	0.25 mg iv	
	Rat	0.3 to 1 mg sc/iv	
	Mouse	0.1 mg sc	
Nicotine acid tartrate	Dog	0.25 mg iv	Ganglion stimulant; higher than 0.3 mg dose produces convulsion
	Cat	0.2 mg iv	
	Rabbit	0.1 mg iv	
	Guinea pig	0.3 mg iv	
	Rat	0.1 mg iv	
Nifedipine	Rat	5 to 50 mg iv	Calcium antagonist; blocks calcium channels
Nikethamide	Dog	75 mg iv	Medullary stimulant
	Rabbit	10 mg iv	
Niludipine	Rat	50 µg iv	Calcium antagonist
Nipecotic acid	Rat	0.5 to 5 mg ip	GABA uptake blocker
NMMA*	Mouse	80 mg po/day for 3 days	NOS inhibitor
Noradrenaline bitartrate or HCl	Dog	0.3 to 5 µg iv	α-Adrenoceptor agonist
	Cat	0.5 to 3 µg iv	
	Rat, Rabbit & Guinea pig	3 µg iv	
Destradiol valerate or benzoate	Rat	0.1 to 1 mg sc	Administered 1 day before experiment
Ouabain octahydrate	Dog	25 µg iv	Slow infusion until arrhythmia sets in
	Cat	2 µg iv	
	Rabbit	50 µg iv	
	Guinea pig	20 µg iv	
Oxotremorine	Rat	0.2 to 0.4 mg ip 0.5 to 2 mg ip	Tremorogenic
	Mouse	2 mg ip	
Oxymetazoline HCl	Rat	0.3 to 3 µg iv	α-Adrenoceptor agonist
Pargyline HCl	Rabbit	25 mg daily for 2 days sc	MAD inhibitor; experiment 12 h after the last dose

\* N<sup>ω</sup>-monomethyl arginine



<i>Drug</i>	<i>Species</i>	<i>Dose per kg body weight and route</i>	<i>Remarks</i>
	Rat	50 mg im	
	Mouse	75 mg ip	3 h before experiment
Paroxetine	Mouse	4 mg ip	5-HT uptake blocker
Pethidine HCl	Rat	30 to 40 mg sc 50 mg ip	Analgesic
Phaclofen	Mouse	10 to 30 mg ip	GABA <sub>A</sub> antagonist
Phenoxybenzamine HCl	Dog	15 mg iv	$\alpha$ -Adrenoceptor blocker; neuronal and extraneuronal CA uptake blocker; 2 h before experiment
	Cat	3 mg iv	
	Rabbit	15 mg iv	
	Rat	1 mg iv	
	Mouse	10 to 20 mg ip	
Phentolamine mesylate or HCl	Dog	1 mg iv	$\alpha$ -Adrenoceptor blocker; 30 min before test
	Cat	1 to 2 mg iv	
	Rabbit	3 mg iv	
	Rat	10 mg iv	
	Mouse	10 mg ip	
Phenylbutazone	Rat	100 to 600 mg po 200 mg sc	Anti-inflammatory agent
Phenylephrine HCl	Rat	5 to 70 $\mu$ g iv total dose	$\alpha$ -Adrenoceptor agonist
Phenylquinone	Mouse	0.25 ml ip (0.02% in 5% alcohol)	Production of writhing syndrome for evaluating analgesics
Phenytoin	Rat	135 mg ip 300 mg sc	Anticonvulsant
Pimozide	Rabbit	4 mg ip	Dopamine (D <sub>1</sub> ) blocker; 3h before experiment
	Rat	10 mg ip	
	Mouse	1 mg ip	
Piperoxan HCl	Rat	20 mg ip	$\alpha$ -Adrenoceptor blocker; 10 min to 1 h before experiment
	Mouse	10 to 20 mg ip	
Prazosin HCl	Rat	0.1 to 1 mg iv 1 mg ip	$\alpha$ -Adrenoceptor blocker; 15 min to 1 h before experiment
	Mouse	20 mg ip	
Propranolol HCl	Rat	15 mg ip 5 mg sc 1 mg iv	$\beta$ -Adrenoceptor blocker; 4 h before experiment

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<i>Drug</i>	<i>Species</i>	<i>Dose per kg body weight and route</i>	<i>Remarks</i>
	Mouse	5 to 10 mg ip	1 h before experiment
Quinidine sulphate	Dog	0.1 to 3 mg iv	Antiarrhythmic
	Cat	2 mg iv	
	Guinea pig	5 mg iv	
Quipazine maleate	Mouse	3 mg ip	5-HT agonist
Reserpine	Dog	0.2 to 1 mg iv	For cardiovascular effects
	Mouse	1 mg ip	
	Rat, Cat & Rabbit	1 mg iv	
	Monkey	0.75 mg im	For behavioral effects: 15 h prior to experiment
	Rabbit	1 mg ip	
	Rat	1 mg im	
	Dog	5 mg sc	For monoamine depletion; 24 h before experiment
	Cat	1 to 5 mg ip/sc	
	Rabbit	5 mg ip/im	
	Guinea pig	0.5 mg ip × 2 days	2 h before experiment
	Rat	5 to 10 mg im/ip	
		0.5 mg ip × 14 days	
		5 to 15 mg ip/sc	20 to 24 h prior to experiment
	Mouse	5 mg ip	
Salbutamol sulphate	Rat	0.5 to 5 mg ip	Selective $\beta_2$ -adrenoceptor agonist
SCH 23390	Rat	2 to 4 mg ip	Dopaminergic (D <sub>1</sub> ) antagonist
SCH 39166	Rat	10 to 20 mg po	Dopaminergic (D <sub>1</sub> ) antagonist
Sertraline	Rat	8 mg iv	5-HT uptake blocker
SKF-525A	Rat	40 mg ip	Hepatic microsomal enzyme inhibitor
SKF-38393	Rat	1 to 4 mg ip 10 mg po	Dopaminergic agonist
Sodium salicylate	Rat	500 mg ip	Anti-inflammatory
Sotalol	Rat	10 mg iv	$\beta_1$ -Adrenoceptor blocker
Spiperone	Rat	2 to 4 mg ip	DA antagonist: 20 min before experiment
	Mouse	0.1 mg ip 1 mg sc	
Streptozotocin	Rat	35 to 65 mg iv	Destroys $\beta$ cells of the islets of Langerhans; use after 2 weeks
	Mouse	125 to 150 mg iv	Use after 7 days

<i>Drug</i>	<i>Species</i>	<i>Dose per kg body weight and route</i>	<i>Remarks</i>
Succinylcholine chloride	Dog	100 mg iv	Depolarizing neuromuscular blocker
	Cat	80 mg iv	
	Rabbit	250 mg iv	
	Rat	450 mg iv	
Sulpiride	Dog	10 mg ip	Dopaminergic antagonist
	Rat	0.1 mg iv	
		0.5 mg ip	
Tetrabenazine methane sulfonate	Rat	25 mg sc	5-HT depletor ; 6 h prior to experiment
	Mouse	30 mg sc	
Thioperamide maleate	Mouse	5 to 20 mg ip	H <sub>3</sub> -antagonist
Tolazoline HCl	Dog	2.5 mg ip	α-Adrenoceptor blocker
	Cat	1 mg ip	
	Rat	10 mg ip	
Tremorine dihydrochloride	Rat & Mouse	5 to 20 mg ip/sc/im	Tremorogenic
Trifluoperazine HCl	Rat	10 to 100 mg sc	To induce catalepsy
Trimethadione	Rabbit	500 mg sc/ip	Antiepileptic
	Rat	40 mg sc	
Tripelennamine (Pyribenzamine) HCl	Dog	5 to 8 mg iv	H <sub>1</sub> -Receptor antagonist
	Cat	5 mg iv	
	Guinea pig	5 mg im/sc	
	Rat	10 mg ip 10 mg iv	
Trypan blue	Rabbit	1 ml of 1% soln. iv	10 to 20 min after intracutaneous administration of test material
d Tubocurarine chloride	Cat & Rabbit	0.12 mg iv	Competitive neuromuscular blocker
U-14624	Rat	200 mg ip	Central depletion of NA more than peripheral
Venlafaxine	Mouse	1 to 8 mg ip	NA uptake blocker
Yohimbine HCl	Rat	1 mg ip	Presynaptic α-receptor blocker; 1 h before experiment 15 min before experiment
	Mouse	1 mg ip	

## GUIDE TO DRUG CONCENTRATIONS FOR ISOLATED PREPARATIONS

<i>Drug (Mol wt)</i>	<i>Preparations</i>	<i>Concentration* (Molar)</i>	<i>Remarks</i>
Acetylcholine chloride (181.7)	Rabbit, ileum	$10^{-7}$ to $2 \times 10^{-6}$	Muscarinic agonist
	ear perfusion	$5 \times 10^{-5}$	
	Guinea pig, ileum	$10^{-8}$ to $10^{-5}$	
	trachea	$2 \times 10^{-7}$ to $2 \times 10^{-8}$	
	Rat, stomach (fundus)	$10^{-7}$ to $10^{-4}$	
	ileum	$10^{-8}$ to $10^{-7}$	
	caecum	$2 \times 10^{-8}$ to $4 \times 10^{-5}$	
	anococcygeus	$10^{-8}$ to $10^{-1}$	
	vas deferens	$10^{-7}$ to $10^{-3}$	
	Mouse ileum	$2 \times 10^{-8}$ to $2 \times 10^{-4}$	
Frog rectus		$10^{-8}$ to $10^{-4}$	Nicotinic agonist
Adenosine triphosphate (507.2)	Guinea pig, ileum	$10^{-8}$ to $10^{-5}$	Purinergetic agonist; produces relaxation or inhibits contraction
	taenia caeci	$10^{-7}$ to $10^{-5}$	
	distal colon	$10^{-8}$ to $10^{-4}$	
	vas deferens	$10^{-8}$ to $5 \times 10^{-5}$	
	left atria	$10^{-5}$	
	Rat, duodenum	$10^{-8}$ to $10^{-5}$	
	vas deferens	$10^{-5}$	
Frog atria	$10^{-5}$		
Adrenaline bitartrate (333.3)	Rabbit, intestine	$10^{-7}$ to $10^{-6}$	Adrenoceptor agonist
	aorta	$10^{-8}$ to $10^{-9}$	
	Langendorff heart	$10^{-6}$ to $3 \times 10^{-4}$	
	Guinea pig, trachea	$10^{-7}$ to $10^{-5}$	
	vas deferens	$10^{-6}$ to $10^{-4}$	
	Rat uterus	$10^{-8}$ to $10^{-8}$	
Angiotensin II amide (1031.2)	Rat, uterus	$10^{-8}$ to $10^{-7}$	Spasmogen
	ascending colon	$2 \times 10^{-9}$	
Apomorphine HCL (312.8)	Rat vas deferens	$10^{-8}$ to $10^{-6}$	Dopamine agonist; inhibits electrically induced contraction
Atropine sulphate (694.8)	Cat trachea	$5 \times 10^{-5}$	Muscarinic antagonist
	Rabbit intestine	$2 \times 10^{-6}$	
	Guinea pig, ileum	$10^{-4}$	
	urinary bladder	$4 \times 10^{-7}$	
	atrium	$2 \times 10^{-6}$	
	Rat, anococcygeus	$10^{-9}$ to $10^{-6}$	
	caecum	$10^{-9}$ to $10^{-7}$	

\*Concentrations are in mole per litre of bath fluid unless indicated otherwise.

<i>Drug (Mol wt)</i>	<i>Preparations</i>	<i>Concentration (Molar)</i>	<i>Remarks</i>
Barium chloride (244.3)	stomach strip	$10^{-7}$	Spasmogen
	vas deferens	$3.4 \times 10^{-6}$	
	Mouse vas deferens	$3 \times 10^{-5}$	
	Rabbit intestine	1 % soln. in Tyrode	
	Guinea pig, ileum trachea	$2 \times 10^{-4}$ to $2 \times 10^{-3}$ $10^{-3}$	
(+) Bicuculline anhydrous (367.3)	Guinea pig colon	$2.7 \times 10^{-7}$ to $2.7 \times 10^{-5}$	GABA antagonist
Bradykinin (1060.2)	Rabbit ear perfusion	$10^{-7}$	Relaxant
	Guinea pig, stomach taenia caeci	$10^{-9}$ to $10^{-8}$ $10^{-9}$ to $10^{-7}$	
	distal colon	$10^{-9}$ to $10^{-7}$	
	Rat duodenum	$10^{-10}$ to $10^{-8}$	
Bretylum tosylate (414.4)	Rabbit ileum	$1.2 \times 10^{-4}$	Adrenergic neuron blocker
	Guinea pig, ileum vas deferens	$10^4$ $2 \times 10^{-5}$	
	taenia caeci	$10^6$	
	Rat, mesenteric artery vas deferens	$2 \times 10^{-5}$ $2 \times 10^{-5}$	
Burimamide (212.3)	Guinea pig atrium	$2 \times 10^{-5}$ to $2 \times 10^{-4}$	$H_2$ -Receptor antagonist
	Rat, uterus	$2 \times 10^{-5}$ to $2 \times 10^{-4}$	
	vas deferens	$2.4 \times 10^{-5}$	
Carbachol (182.6)	Cat trachea	$10^{-9}$ to $10^{-4}$	Cholinergic agonist
	Rabbit intestine	0.001% soln. in Tyrode	
	Guinea pig ileum	$10^{-9}$ to $10^{-5}$	
	Rat, caecum	$2 \times 10^{-8}$ to $2 \times 10^{-6}$	
	uterus	$3 \times 10^{-6}$ to $5 \times 10^{-6}$	
	Frog rectus	$5 \times 10^{-9}$ to $5 \times 10^{-7}$	
Cimetidine (252.3)	Guinea pig right atrium	$3 \times 10^5$ to $10^{-4}$	$H_2$ -Receptor antagonist
	Rat stomach	$10^4$ to $10^{-3}$	
Clobenpropit dihydrobromide (470.7)	Guinea pig ileum	$10^{-5}$ to $3 \times 10^{-5}$	$H_3$ -antagonist
Clonidine HCl (266.6)	Rabbit heart	$4.3 \times 10^{-8}$	$\alpha_2$ -Adrenoceptor agonist
	Guinea pig, ileum	$10^{-8}$ to $10^{-5}$	
	urinary bladder	$3 \times 10^{-8}$	

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<i>Drug (Mol wt)</i>	<i>Preparations</i>	<i>Concentration (Molar)</i>	<i>Remarks</i>
Cocaine HCl (339.8)	Rat, thoracic aorta vas deferens	$4 \times 10^{-8}$ $10^{-10}$ to $10^{-8}$	Neuronal catecholamine uptake inhibitor
	Rabbit, aorta ileum	$10^{-5}$ $3 \times 10^{-5}$	
	splenic strip vas deferens	$3 \times 10^{-5}$ $1.8 \times 10^{-5}$	
	Rat vas deferens	$1.8 \times 10^{-5}$	
Corticosterone (346.4)	Rabbit ileum & splenic strip	$2.8 \times 10^{-5}$	Extraneuronal catecholamine uptake inhibitor
	Guinea pig ileum	$3 \times 10^{-5}$	
Cyproheptadine HCl (350.9)	Rat anococcygeus	$10^{-6}$	Serotonin antagonist
Desipramine HCl (302.8)	Guinea pig vas deferens & atria	$10^{-8}$ to $10^{-7}$	Neuronal catecholamine uptake inhibitor
	Rat vas deferens	$10^{-7}$	
Desoxycorticosterone acetate (372.5)	Guinea pig trachea	$5 \times 10^{-6}$ to $2 \times 10^{-5}$	Extraneuronal catecholamine uptake inhibitor
	Rat heart	$3 \times 10^{-5}$	
Dexamethasone diso- dium phosphate (516.4)	Guinea pig fundus	$10^{-5}$	NOS* inhibitor
Diphenhydramine HCl (291.8)	Rat stomach strip	$7 \times 10^{-7}$	H <sub>1</sub> -Receptor antagonist
Disodium edetate (372.2)	Rabbit, ear perfusion ileum	$10^{-5}$ $3 \times 10^{-5}$	Chelating agent; prevents oxidative degradation of catecholamines
	splenic strip	$3 \times 10^{-5}$	
DMPP** (318.2)	Rabbit heart	$2 \times 10^{-8}$ to $2 \times 10^{-7}$	Ganglionic stimulant
	Guinea pig intestine	$1.3 \times 10^{-5}$	
	Rat vas deferens	$10^{-7}$ to $10^{-4}$	
Dopamine HCl (189.6)	Rat vas deferens	$10^{-8}$ to $10^{-6}$	Inhibition of electrical contraction
Epibatidine dihydrochloride (281.6)	Guinea pig ileum	$10^{-9}$ to $10^{-8}$	N***-agonist

\* Nitric oxide synthases

\*\* 1,1-Dimethyl-4-phenyl piperazinium iodide

\*\*\* Nicotinic

<i>Drug (Mol wt)</i>	<i>Preparations</i>	<i>Concentration (Molar)</i>	<i>Remarks</i>
Ergometrine maleate (441.5)	Rat uterus	$1.1 \times 10^{-6}$ to $1.1 \times 10^{-3}$	Oxytocic agent
Flumazenil (303.3)	Rat trachea	$10^{-6}$ to $10^{-4}$	GABA <sub>A</sub> - antagonist
Gamma-aminobutyric acid (GABA) (103.1)	Guinea pig colon	$3 \times 10^{-4}$	Relaxation
Guanethidine sulphate (494.7)	Rabbit ileum Guinea pig, urinary bladder stomach Rat vas deferens	$2 \times 10^{-5}$ $10^{-6}$ $2 \times 10^{-5}$ $10^{-6}$ to $10^{-5}$	Adrenergic neuron blocker
Haloperidol (375.9)	Rat vas deferens	$10^{10}$ to $10^8$	Dopamine antagonist
Hemicholinium-3 (574.4)	Guinea pig ileum	$2 \times 10^{-5}$	ACh synthesis blocker
Hexahydroisiladifenidol (367.1)	Rat heart	$3 \times 10^{-7}$	M <sub>3</sub> -antagonist
Hexamethonium bromide (362.2)	Guinea pig, ileum taenia caecum Rat, vas deferens anococcygeus	$10^{-5}$ $10^6$ $10^6$ $10^{-4}$	Ganglion blocker
Histamine acid phosphate (325.2)	Guinea pig, tracheal chain atrium Rat vas deferens	$10^{-7}$ to $10^{-4}$ $10^{-7}$ to $10^{-3}$ $10^{-7}$ to $10^{-4}$	H-Receptor agonist
Hydrocortisone hemisuccinate sodium (484.5)	Guinea pig trachea	$5 \times 10^{-5}$	Extraneuronal catecholamine uptake inhibitor
5-Hydroxytryptamine (serotonin) creatinine sulphate (387.5)	Cat trachea Rabbit heart Guinea pig ileum  Rat, vas deferens anococcygeus fundus caecum	$10^{-9}$ to $10^{-5}$ $10^{-9}$ to $2 \times 10^{-7}$ $2.5 \times 10^9$ to $2.5 \times 10^{-5}$ $10^{-7}$ to $10^{-5}$ $10^{-7}$ to $10^{-5}$ $10^{-8}$ to $10^{-5}$ $10^9$ to $10^{-5}$	Tryptaminergic agonist
Hyoscine HBr (438.3)	Guinea pig, ileum taenia caeci	$5 \times 10^{-7}$ $1.3 \times 10^{-6}$	Muscarinic blocker

<i>Drug (Mol wt)</i>	<i>Preparations</i>	<i>Concentration (Molar)</i>	<i>Remarks</i>
Imetit dihydrobromide (332.1)	Rat aorta	$3 \times 10^{-6}$	H <sub>3</sub> -agonist
Imipramine HCl (316.9)	Rat anococcygeus	$10^{-6}$	Neuronal catecholamine uptake inhibitor
Indomethacin (357.8)	Guinea pig, trachea	$1.7 \times 10^{-6}$	Prostaglandin synthetase inhibitor
	stomach	$10^{-5}$ to $10^{-4}$	
	urinary bladder	$5 \times 10^{-6}$	
	Rat, vas deferens	$1.4 \times 10^{-5}$	
	urinary bladder	$5 \times 10^{-6}$	
Iproniazid phosphate (277.2)	Rabbit aorta	$2 \times 10^{-4}$	Monoamine oxidase inhibitor
	Rabbit, guinea pig & rat splenic strips	$3.6 \times 10^{-4}$	
Isoprenaline HCl (247.7) sulphate (556.6)	Guinea pig, taenia caeci	$10^{-6}$ to $10^{-4}$	$\beta$ -Adrenoceptor agonist
	atrium	$4 \times 10^{-6}$	
	ileum	$2 \times 10^{-6}$	
	vas deferens	$2 \times 10^{-6}$	
	stomach	$10^{-6}$ to $5 \times 10^{-5}$	
	urinary bladder	$5 \times 10^{-6}$	
	Rat, vas deferens	$3.5 \times 10^{-6}$	
	uterus	$5 \times 10^{-7}$	
	anococcygeus	$10^{-6}$	
	caecum	$3 \times 10^{-9}$ to $10^{-7}$	
Mepyramine (Pyrilamine) maleate (401.5)	Cat trachea	$10^{-7}$	H <sub>1</sub> -Receptor antagonist
	Guinea pig trachea & ileum	$10^{-6}$	
Metanephrine HCl (233.7)	Guinea pig trachea & atria	$10^5$ to $10^4$	Extraneuronal catecholamine uptake inhibitor
Methoxamine HCl (247.7)	Guinea pig vas deferens	$10^6$ to $10^3$	$\alpha_1$ -Adrenoceptor agonist
	Rat mesenteric artery	$4 \times 10^6$ to $4 \times 10^5$	
$\alpha$ -Methyl histamine dihydrochloride (198.1)	Guinea pig ileum	$10^{-4}$	H <sub>3</sub> -agonist
Methysergide bimalate (469.5)	Cat trachea	$10^6$	Serotonin antagonist
	Rat, vas deferens	$2.8 \times 10^7$	
	anococcygeus	$2 \times 10^6$ to $10^6$	
	stomach strip	$6 \times 10^7$	
	ascending colon	$10^6$	
Morphine HCl (375.8)	Guinea pig ileum	$10^{-6}$	Inhibits electrical contraction
	Mouse vas deferens	$10^{-8}$	



<i>Drug (Mol wt)</i>	<i>Preparations</i>	<i>Concentration (Molar)</i>	<i>Remarks</i>
Naloxone HCl (363.8)	Guinea pig ileum Mouse vas deferens	$10^{-9}$ $10^{-7}$ to $10^{-8}$	Opiate antagonist
Neostigmine bromide (303.2)	Guinea pig ileum Rat anococcygeus Frog rectus	$2 \times 10^{-7}$ $10^{-6}$ $2 \times 10^{-5}$	Cholinoceptor agonist
Nialamide (298.3)	Rat anococcygeus	$2.2 \times 10^{-6}$	Monoamine oxidase inhibitor
Nicotine bitartrate (498.4)	Guinea pig ileum	$3 \times 10^{-6}$ to $3 \times 10^{-5}$	Ganglionic stimulant
Nifedipine (346.3)	Guinea pig heart Rat, vas deferens thoracic aorta	$10^8$ to $10^{-6}$ $10^6$ $10^7$	Calcium antagonist
NMMA* (248.3)	Rat aorta	$10^{-4}$	NOS inhibitor
Noradrenaline bitartrate (337.3)	Rabbit, heart aorta duodenum Guinea pig, vas deferens taenia caeci Rat, thoracic aorta & portal vein vas deferens uterus caecum mesenteric artery	$10^9$ to $10^{-8}$ $10^8$ $10^3$ $10^{-8}$ to $10^{-3}$ $10^{-9}$ to $10^7$ $10^{-11}$ to $10^{-8}$ $10^{-8}$ to $10^{-4}$ $10^{-6}$ $10^{-8}$ to $10^6$ $10^{-7}$	$\alpha$ -Adrenoceptor agonist
17, $\beta$ -Oestradiol (272.4)	Rabbit, guinea pig & rat splenic strips Guinea pig atria	$7.3 \times 10^7$ $5 \times 10^{-6}$	Extraneuronal catecholamine uptake inhibitor
Ouabain octahydrate (728.8)	Rabbit atria Guinea pig ileum Frog heart	$5 \times 10^{-6}$ $10^{-8}$ to $10^{-4}$ $10^{-6}$ to $4 \times 10^{-6}$	Inhibits Na-K ATPase activity
Oxprenolol HCl (301.8)	Rat ascending colon	$3.3 \times 10^{-5}$	$\beta$ -Adrenoceptor blocker
Oxytocin (1007.2)	Rat uterus	$2 \times 10^{-9}$ to $2 \times 10^{-8}$	Oxytocic

\* N<sup>G</sup> – monomethyl arginine

<i>Drug (Mol wt)</i>	<i>Preparations</i>	<i>Concentration (Molar)</i>	<i>Remarks</i>
Papaverine HCl (375.8)	Rabbit, intestine	$2 \times 10^{-4}$	Smooth muscle relaxant
	ear perfusion	$10^{-4}$	
	Guinea pig ileum	$10^{-7}$	
	Rat uterus	$3 \times 10^{-6}$	
Phaclofen (249.6)	Rat trachea	$10^{-5}$	GABA <sub>B</sub> antagonist
Phenoxybenzamine HCl (340.3)	Rabbit, guinea pig splenic strips	$10^{-8}$ to $4 \times 10^{-9}$	Neuronal catecholamine uptake inhibitor; also extraneuronal catecholamine uptake inhibitor in high dose
	Guinea pig, atrium trachea	$10^{-5}$ $5 \times 10^{-5}$	
	Rat splenic strip	$10^{-6}$	
Phentolamine mesylate (377.5)	Guinea pig, atrium ileum	$4 \times 10^{-6}$ $2 \times 10^{-6}$	$\alpha$ -Adrenoceptor blocker
	stomach	$5 \times 10^{-6}$ to $5 \times 10^{-5}$	
	trachea	$10^{-5}$	
	vas deferens	$2.5 \times 10^{-5}$	
	urinary bladder	$5 \times 10^{-6}$	
	Rat, anococcygeus uterus	$10^{-9}$ to $10^{-6}$ $5 \times 10^{-7}$	
	vas deferens	$3.5 \times 10^{-6}$	
	caecum	$10^{-7}$ to $10^{-5}$	
	stomach strip	$7 \times 10^{-7}$	
l-Phenylephrine HCl (203.7)	Rat thoracic aorta	$3 \times 10^{-8}$	$\alpha$ -Adrenoceptor agonist
Physostigmine (Eserine) salicylate (413.4) sulphate (648.8)	Rabbit ileum	$10^{-5}$	Cholinoceptor agonist
	Guinea pig, ileum	$2 \times 10^{-6}$	
	urinary bladder	$2 \times 10^{-6}$	
	Rat caecum	$8 \times 10^{-7}$	
	Frog rectus	$2 \times 10^{-5}$ to $4 \times 10^{-5}$	
Pimozide (461.6)	Rat vas deferens	$4 \times 10^{-9}$ to $2.5 \times 10^{-7}$	Dopamine antagonist
Pindolol (248.3)	Guinea pig, atrium	$10^{-5}$ to $10^{-3}$	$\beta$ -Adrenoceptor blocker
	vas deferens	$10^{-3}$ to $10^{-2}$	
Polyphloretin phosphate	Rabbit jejunum & uterus	$2.5$ to $30 \mu\text{g/ml}$	Prostaglandin antagonist
	Guinea pig ileum	$50$ to $300 \mu\text{g/ml}$	
	Rat, uterus	$10$ to $50 \mu\text{g/ml}$	
	ascending colon	$20 \mu\text{g/ml}$	
Potassium chloride (74.6)	Guinea pig ileum	$6 \times 10^{-2}$	Spasmogen
	Rat mesenteric artery	$10^{-3}$ to $1.5 \times 10^{-2}$	

Drug (Mol wt)	Preparations	Concentration (Molar)	Remarks
	Mouse, ileum vas deferens	$4 \times 10^{-3}$ $4 \times 10^{-3}$	
Practolol (266.3)	Guinea pig atrium	$10^{-7}$ to $4 \times 10^{-3}$	$\beta$ -Adrenoceptor blocker
Prazosin HCl (419.9)	Rat, vas deferens thoracic aorta	$2 \times 10^{-5}$ $10^{-10}$ to $10^{-9}$	$\alpha_1$ -Adrenoceptor blocker
Promethazine HCl (320.9)	Guinea pig, left atrium ileum	$3 \times 10^{-5}$ $10^{-6}$	$H_1$ -Receptor blocker
d, l-Propranolol HCl (295.8)	Rabbit, heart ileum splenic strip Guinea pig, atrium urinary bladder splenic strip Rat, caecum splenic strip uterus vas deferens stomach strip	$3 \times 10^{-6}$ $4 \times 10^{-6}$ to $10^{-5}$ $1.7 \times 10^{-6}$ $4 \times 10^{-6}$ $5 \times 10^{-6}$ $1.7 \times 10^{-6}$ $10^{-7}$ to $10^{-6}$ $1.7 \times 10^{-6}$ $10^{-6}$ $1.9 \times 10^{-5}$ $7 \times 10^{-7}$	$\beta$ -Adrenoceptor blocker
Prostacycline sodium (374.4)	Guinea pig, stomach lung strips	$10^{-10}$ to $10^{-6}$ $10^{-9}$ to $10^{-5}$	Contraction
Prostaglandin $E_2$ (352.5)	Guinea pig longitudinal muscle Rat, mesenteric artery uterus aorta	$10^{-9}$ to $10^{-4}$ $10^{-6}$ to $3 \times 10^{-5}$ $9 \times 10^{-5}$ $9 \times 10^{-4}$	Contraction
Prostaglandin $F_{2\alpha}$ (354.5)	Rat, mesenteric artery aorta	$10^{-5}$ $3 \times 10^{-5}$	Contraction
Pyrenzepine dihydrochloride (424.3)	Guinea pig ileum	$10^{-8}$ to $10^{-6}$	$M_1$ -antagonist
Pyrogallol (126.1)	Rat anococcygeus	$2.3 \times 10^{-5}$	Catechol-O-methyltransferase (COMT) blocker
SC-19220 (336.0)	Rabbit ear perfusion Guinea pig ileum Rat stomach	$10^{-5}$ $3 \times 10^{-6}$ $5 \times 10^{-6}$	PG-Receptor antagonist

## Appendix

<i>Drug (Mol wt)</i>	<i>Preparations</i>	<i>Concentration (Molar)</i>	<i>Remarks</i>
Sotalol HCl (308.8)	Rabbit heart	$4 \times 10^{-6}$	$\beta$ -Adrenoceptor blocker
	Guinea pig, atrium ileum	$4 \times 10^{-6}$ $5 \times 10^{-5}$	
Succinylcholine chloride (397.3)	Frog rectus	$10^{-5}$	Neuromuscular blocker
Tetrodotoxin anhydrous (319.3)	Guinea pig, ileum	$5 \times 10^{-7}$	Inhibition of axonal conduction
	stomach	$5 \times 10^{-3}$	
	vas deferens	$3 \times 10^{-4}$	
	urinary bladder	$5 \times 10^{-7}$	
	taenia coli	$10^{-6}$	
Rat anococcygeus	$2 \times 10^{-7}$		
Tropolone (122.1)	Rabbit ileum, splenic & aortic strips	$3 \times 10^{-5}$	COMT blocker
d-Tubocurarine chloride	Rat phrenic nerve- (695.66)		Neuromuscular blocker diaphragm $10^6$ to $10^4$
	Frog rectus	$10^6$ to $7 \times 10^6$	
Verapamil HCl (491.1)	Rabbit ear perfusion	$5 \times 10^{-6}$ to $10^{-5}$	Calcium antagonist
	Guinea pig, ileum heart	$10^{-4}$ $10^{-7}$ to $10^{-6}$	
	Rat, thoracic aorta & portal vein	$10^7$ to $10^6$	
	fundus	$10^6$ to $10^{-5}$	
Yohimbine HCl (390.9)	Rabbit heart	$2.8 \times 10^{-6}$	$\alpha$ -Adrenoceptor blocker
	Guinea pig ileum	$10^4$ to $5 \times 10^{-3}$	
	Rat, vas deferens	$10^{-7}$ to $10^{-5}$	
	thoracic aorta	$5 \times 10^{-8}$ to $10^{-7}$	

TABLE OF RANDOM NUMBER

	00 0	05-09	10-14	15-19	20-24	25-29	30-34	35-39	40-44	45-49
00	54463	22662	65905	7639	79365	67382	29085	69831	47058	08186
01	15389	85205	18850	39226	42249	90669	96325	23248	60933	26927
02	85941	40756	82414	02015	13858	78030	16269	65978	01385	15345
03	61149	69440	11286	88218	58925	03638	52862	62733	33451	77455
04	05219	81619	10651	67079	92511	59888	84502	72095	83463	75577
05	41417	98326	87719	92294	46614	50948	64886	20002	97365	30976
06	28357	94070	20652	35774	16249	75019	21145	05217	47286	76305
07	17783	00015	10806	83091	91530	36466	39981	62481	49177	75779
08	40950	84820	29881	85966	62800	70326	84740	62660	77379	90279
09	82995	64157	66164	41180	10089	41757	78258	96488	88629	37231
10	96754	17676	55659	44105	47361	34833	86679	23930	53249	27083
11	34357	88040	53364	71726	45690	66334	60332	22554	90600	71113
12	06318	37403	49927	57715	50423	67372	63116	48888	21505	80182
13	62211	52820	07243	79931	89292	84767	85693	73947	22278	11551
14	47534	09243	67879	00544	23410	12740	02540	54440	32949	13491
15	98614	75993	84460	62846	59844	14922	48730	73443	48167	34770
16	24856	03648	44898	09351	98795	18644	39765	71058	90368	44104
17	96887	12479	80621	66223	86085	78285	02432	53342	42846	94771
18	90801	21472	42815	77408	37390	76766	52615	32141	30268	18106
19	55165	77312	83666	36028	28420	70219	81369	41943	47366	41067
20	75884	12952	84318	95108	72305	64620	91318	89872	45375	85436
21	16777	37116	58550	42958	21460	43910	01175	87894	81378	10620
22	46230	43877	80207	88877	89380	32992	91380	03164	98656	59337
23	42902	66892	46134	10432	94710	23474	20423	60137	60609	13119
24	81007	00333	39693	28039	10154	95425	39220	19774	31782	49037
25	68089	01122	51111	72373	06902	74373	96199	97017	41273	21546
26	20411	67081	89950	16944	93054	87687	96693	87236	77054	33848
27	58212	13160	06468	15718	82627	76999	05999	58680	96739	63700
28	70577	42866	24969	61210	76046	67699	42054	12696	93758	03283
29	94522	74358	71659	62038	79643	79169	44741	05437	39038	13163
30	42626	86819	85651	88678	17401	03252	99547	32404	17918	62880
31	16051	33763	57194	16752	54450	19031	58580	47629	54132	60631
32	08244	27647	33851	44705	94211	46716	11738	55784	95374	72655
33	59497	04392	09419	89964	51211	04894	72882	17805	21896	83864
34	97155	13428	40293	09985	58434	01412	69124	82171	59058	82859
35	98409	66162	95763	47420	20792	61527	20441	39435	11859	41567
36	45476	84882	65109	96597	25930	66790	65706	61203	53634	22557
37	89300	69700	50741	30379	11658	23166	05400	66669	48708	03887
38	50051	95137	91631	66315	91428	12275	24816	68091	71710	33258
39	31753	85178	31310	89642	98364	02306	24617	09609	83942	22716
40	79152	53829	77250	20190	56535	18760	69942	77448	33278	48805
41	44560	38750	83635	56540	64900	42912	13953	79149	18710	68618
42	68328	83378	63369	71381	39564	05615	42451	64559	97501	65747
43	46939	38689	58625	08342	30459	85863	20781	09284	26333	91777
44	83544	86141	15707	96256	23068	13782	08467	89469	93842	55349

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DF	Probability					
	0.5	0.1	0.05	0.02	0.01	0.001
1	1.000	6.314	12.706	31.821	63.657	636.619
2	0.816	2.920	4.303	6.965	9.925	31.598
3	0.765	2.353	3.182	4.541	5.841	12.941
4	0.741	2.132	2.776	3.747	4.604	8.610
5	0.727	2.015	2.571	3.365	4.032	6.859
6	0.718	1.943	2.447	3.143	3.707	5.959
7	0.711	1.895	2.365	2.998	3.499	5.405
8	0.706	1.860	2.306	2.896	3.355	5.041
9	0.703	1.833	2.262	2.821	3.250	4.781
10	0.700	1.812	2.228	2.764	3.169	4.587
11	0.697	1.796	2.201	2.718	3.106	4.437
12	0.695	1.782	2.179	2.681	3.055	4.318
13	0.694	1.771	2.160	2.650	3.012	4.221
14	0.692	1.761	2.145	2.624	2.977	4.140
15	0.691	1.753	2.131	2.602	2.947	4.073
16	0.690	1.746	2.120	2.583	2.921	4.015
17	0.689	1.740	2.110	2.567	2.898	3.965
18	0.688	1.734	2.101	2.552	2.878	3.922
19	0.688	1.729	2.093	2.539	2.861	3.883
20	0.687	1.725	2.086	2.528	2.845	3.850
21	0.686	1.721	2.080	2.518	2.831	3.819
22	0.686	1.717	2.074	2.506	2.819	3.792
23	0.685	1.714	2.069	2.500	2.807	3.767
24	0.685	1.711	2.064	2.492	2.797	3.745
25	0.684	1.708	2.060	2.485	2.787	3.725
26	0.684	1.706	2.056	2.479	2.779	3.707
27	0.684	1.703	2.052	2.473	2.771	3.690
28	0.683	1.701	2.048	2.467	2.763	3.674
29	0.683	1.699	2.045	2.462	2.756	3.659
30	0.683	1.697	2.042	2.457	2.750	3.646
40	0.681	1.684	2.021	2.423	2.704	3.551
60	0.679	1.671	2.000	2.390	2.660	3.460
120	0.677	1.658	1.980	2.358	2.617	3.373
$\infty$	0.674	1.645	1.960	2.326	2.576	3.291

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TABLE OF DISTRIBUTION OF F

P = 0.05

n <sub>2</sub>	n <sub>1</sub> degrees of freedom (for greater mean square)										
	1	2	3	4	5	6	7	8	12	24	∞
5	6.61	5.79	5.41	5.19	5.05	4.95	4.88	4.82	4.68	4.53	4.37
6	5.99	5.14	4.76	4.53	4.39	4.28	4.21	4.15	4.00	3.84	3.67
7	5.59	4.74	4.35	4.12	3.97	3.87	3.79	3.73	3.57	3.41	3.23
8	5.32	4.46	4.07	3.84	3.69	3.58	3.50	3.44	3.28	3.12	2.93
9	5.12	4.26	3.86	3.63	3.48	3.37	3.29	3.23	3.07	2.90	2.71
10	4.96	4.10	3.71	3.48	3.33	3.22	3.14	3.07	2.91	2.74	2.54
11	4.84	3.98	3.59	3.36	3.20	3.09	3.01	2.95	2.79	2.61	2.41
12	4.75	3.89	3.49	3.26	3.11	3.00	2.91	2.85	2.69	2.51	2.30
13	4.67	3.81	3.41	3.18	3.03	2.92	2.83	2.77	2.60	2.42	2.21
14	4.60	3.74	3.34	3.11	2.96	2.85	2.76	2.70	2.53	2.35	2.13
15	4.54	3.68	3.29	3.06	2.90	2.79	2.71	2.64	2.48	2.29	2.07
16	4.49	3.63	3.24	3.01	2.85	2.74	2.66	2.59	2.42	2.24	2.01
17	4.45	3.59	3.20	2.96	2.81	2.70	2.61	2.55	2.38	2.19	1.96
18	4.41	3.55	3.16	2.93	2.77	2.66	2.58	2.51	2.34	2.15	1.92
19	4.38	3.52	3.13	2.90	2.74	2.63	2.54	2.48	2.31	2.11	1.88
20	4.35	3.49	3.10	2.87	2.71	2.60	2.51	2.45	2.28	2.08	1.84
21	4.32	3.47	3.07	2.84	2.68	2.57	2.49	2.42	2.25	2.05	1.81
22	4.30	3.44	3.05	2.82	2.66	2.55	2.46	2.40	2.23	2.03	1.78
23	4.28	3.42	3.03	2.80	2.64	2.53	2.44	2.37	2.20	2.01	1.76
24	4.26	3.40	3.01	2.78	2.62	2.51	2.42	2.36	2.18	1.98	1.73
25	4.24	3.39	2.99	2.76	2.60	2.49	2.40	2.34	2.16	1.96	1.71
26	4.23	3.37	2.98	2.74	2.59	2.47	2.39	2.32	2.15	1.95	1.69
27	4.21	3.35	2.96	2.73	2.57	2.46	2.37	2.31	2.13	1.93	1.67
28	4.20	3.34	2.95	2.71	2.56	2.45	2.36	2.29	2.12	1.91	1.66
29	4.18	3.33	2.93	2.70	2.55	2.43	2.35	2.28	2.10	1.90	1.64
30	4.17	3.32	2.92	2.69	2.53	2.42	2.33	2.27	2.09	1.89	1.62
40	4.08	3.23	2.84	2.61	2.45	2.34	2.25	2.18	2.00	1.79	1.51
60	4.00	3.15	2.76	2.53	2.37	2.25	2.17	2.10	1.92	1.70	1.39
80	3.96	3.11	2.72	2.49	2.33	2.21	2.13	2.06	1.88	1.65	1.33
100	3.94	3.09	2.70	2.46	2.31	2.19	2.10	2.03	1.85	1.63	1.28
120	3.92	3.07	2.68	2.45	2.29	2.18	2.09	2.02	1.83	1.61	1.26
∞	3.84	3.00	2.61	2.37	2.22	2.10	2.01	1.94	1.75	1.52	1.00

TABLE OF DISTRIBUTION OF F  
P = 0.01

$n_2$	$n_1$ degrees of freedom (for greater mean square)										
	1	2	3	4	5	6	7	8	12	24	∞
5	16.26	13.27	12.06	11.39	10.97	10.67	10.46	10.29	9.89	9.47	9.02
6	13.75	10.92	9.78	9.15	8.75	8.47	8.26	8.10	7.72	7.31	6.88
7	12.25	9.55	8.45	7.85	7.46	7.19	6.99	6.84	6.47	6.07	5.65
8	11.26	8.65	7.59	7.01	6.63	6.37	6.18	6.03	5.67	5.28	4.86
9	10.56	8.02	6.99	6.42	6.06	5.80	5.61	5.47	5.11	4.73	4.31
10	10.04	7.56	6.55	5.99	5.64	5.39	5.20	5.06	4.71	4.33	3.91
11	9.65	7.21	6.22	5.67	5.32	5.07	4.89	4.74	4.40	4.02	3.60
12	9.33	6.93	5.95	5.41	5.06	4.82	4.64	4.50	4.16	3.78	3.36
13	9.07	6.70	5.74	5.21	4.86	4.62	4.44	4.30	3.96	3.59	3.17
14	8.86	6.51	5.56	5.04	4.69	4.32	4.28	4.14	3.80	3.43	3.01
15	8.68	6.36	5.42	4.89	4.56	4.32	4.14	4.00	3.67	3.29	2.87
16	8.53	6.23	5.29	4.77	4.44	4.20	4.03	3.89	3.55	3.18	2.75
17	8.40	6.11	5.18	4.67	4.34	4.10	3.93	3.79	3.46	3.08	2.65
18	8.29	6.01	5.09	4.58	4.25	4.01	3.84	3.71	3.37	3.00	2.57
19	8.18	5.93	5.01	4.50	4.17	3.94	3.77	3.63	3.30	2.92	2.49
20	8.10	5.85	4.94	4.43	4.10	3.87	3.70	3.56	3.23	2.86	2.42
21	8.02	5.78	4.87	4.37	4.04	3.81	3.64	3.51	3.17	2.80	2.36
22	7.95	5.72	4.82	4.31	3.99	3.76	3.59	3.45	3.12	2.75	2.31
23	7.88	5.66	4.76	4.26	3.94	3.71	3.54	3.41	3.07	2.70	2.26
24	7.82	5.61	4.72	4.22	3.90	3.67	3.50	3.36	3.03	2.66	2.21
25	7.77	5.57	4.68	4.18	3.85	3.63	3.46	3.32	2.99	2.62	2.17
26	7.72	5.53	4.64	4.14	3.82	3.59	3.42	3.29	2.96	2.58	2.13
27	7.68	5.49	4.60	4.11	3.78	3.56	3.39	3.26	2.93	2.55	2.10
28	7.64	5.45	4.57	4.07	3.75	3.53	3.36	3.23	2.90	2.52	2.07
29	7.60	5.42	4.54	4.04	3.73	3.50	3.33	3.20	2.87	2.49	2.04
30	7.56	5.39	4.51	4.02	3.70	3.47	3.30	3.17	2.84	2.47	2.01
40	7.31	5.18	4.31	3.83	3.51	3.29	3.12	2.99	2.66	2.29	1.81
60	7.08	4.98	4.13	3.65	3.34	3.12	2.95	2.82	2.50	2.12	1.60
80	6.96	4.88	4.04	3.56	3.26	3.04	2.87	2.74	2.42	2.03	1.50
100	6.90	4.82	3.98	3.51	3.21	2.99	2.82	2.69	2.37	1.98	1.43
120	6.85	4.79	3.95	3.48	3.17	2.96	2.79	2.66	2.34	1.95	1.38
∞	6.64	4.61	3.78	3.32	3.02	2.80	2.64	2.51	2.19	1.79	1.00



DUNNETT TABLE

Number of Groups Including Control Group

dfc	alpha	2	3	4	5	6	7	8	9	10
5	0.05	2.57	3.03	3.29	3.48	3.62	3.73	3.82	3.9	3.97
	0.01	4.03	4.63	4.98	5.22	5.41	5.56	5.69	5.8	5.89
6	0.05	2.45	2.86	3.1	3.26	3.39	3.49	3.57	3.64	3.71
	0.01	3.71	4.21	4.51	4.71	4.87	5	5.1	5.2	5.28
7	0.05	2.36	2.75	2.97	3.12	3.24	3.33	3.41	3.47	3.53
	0.01	3.5	3.95	4.21	4.39	4.53	4.64	4.74	4.82	4.89
8	0.05	2.31	2.67	2.88	3.02	3.13	3.22	3.29	3.35	3.41
	0.01	3.36	3.77	4	4.17	4.29	4.4	4.48	4.56	4.62
9	0.05	2.26	2.61	2.81	2.95	3.05	3.14	3.2	3.26	3.32
	0.01	3.25	3.63	3.85	4.01	4.12	4.22	4.3	4.37	4.43
10	0.05	2.23	2.57	2.76	2.89	2.99	3.07	3.14	3.19	3.24
	0.01	3.17	3.53	3.74	3.88	3.99	4.08	4.16	4.22	4.28
11	0.05	2.2	2.53	2.72	2.84	2.94	3.02	3.08	3.14	3.19
	0.01	3.11	3.45	3.65	3.79	3.89	3.98	4.05	4.11	4.16
12	0.05	2.18	2.5	2.68	2.81	2.9	2.98	3.04	3.09	3.14
	0.01	3.05	3.39	3.58	3.71	3.81	3.89	3.96	4.02	4.07
13	0.05	2.16	2.48	2.65	2.78	2.87	2.94	3	3.06	3.1
	0.01	3.01	3.33	3.52	3.65	3.74	3.82	3.89	3.94	3.99
14	0.05	2.14	2.46	2.63	2.75	2.84	2.91	2.97	3.02	3.07
	0.01	2.98	3.29	3.47	3.59	3.69	3.76	3.83	3.88	3.93
15	0.05	2.13	2.44	2.61	2.73	2.82	2.89	2.95	3	3.04
	0.01	2.95	3.25	3.43	3.55	3.64	3.71	3.78	3.83	3.88
16	0.05	2.12	2.42	2.59	2.71	2.8	2.87	2.92	2.97	3.02
	0.01	2.92	3.22	3.39	3.51	3.6	3.67	3.73	3.78	3.83
17	0.05	2.11	2.41	2.58	2.69	2.78	2.85	2.9	2.95	3
	0.01	2.9	3.19	3.36	3.47	3.56	3.63	3.69	3.74	3.79
18	0.05	2.1	2.4	2.56	2.68	2.76	2.83	2.89	2.94	2.98
	0.01	2.88	3.17	3.33	3.44	3.53	3.6	3.66	3.71	3.75
19	0.05	2.09	2.39	2.55	2.66	2.75	2.81	2.87	2.92	2.96
	0.01	2.86	3.15	3.31	3.42	3.5	3.57	3.63	3.68	3.72
20	0.05	2.09	2.38	2.54	2.65	2.73	2.8	2.86	2.9	2.95
	0.01	2.85	3.13	3.29	3.4	3.48	3.55	3.6	3.65	3.69
24	0.05	2.06	2.35	2.51	2.61	2.7	2.76	2.81	2.86	2.9
	0.01	2.8	3.07	3.22	3.32	3.4	3.47	3.52	3.57	3.61
30	0.05	2.04	2.32	2.47	2.58	2.66	2.72	2.77	2.82	2.86
	0.01	2.75	3.01	3.15	3.25	3.33	3.39	3.44	3.49	3.52
40	0.05	2.02	2.29	2.44	2.54	2.62	2.68	2.73	2.77	2.81
	0.01	2.7	2.95	3.09	3.19	3.26	3.32	3.37	3.41	3.44
60	0.05	2	2.27	2.41	2.51	2.58	2.64	2.69	2.73	2.77
	0.01	2.66	2.9	3.03	3.12	3.19	3.25	3.29	3.33	3.37

**Table**

**TABLE OF WILCOXON TEST ON PAIRED SAMPLES**

Number of pairs	5% Level	1% Level
7	2	0
8	2	0
9	6	2
10	8	3
11	11	5
12	14	7
13	17	10
14	21	13
15	25	16
16	30	19

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**TABLE OF WILCOXON TEST ON UNPAIRED SAMPLES**

5% Critical points of rank sums

$n_2$ ↓	$n_1$ →	2	3	4	5	6	7	8	9	10	11	12	13	14	15
4				10											
5			6	11	17										
6			7	12	18	26									
7			7	13	20	27	36								
8	3	8	14	21	29	38	49								
9	3	8	15	22	31	40	51	63							
10	3	9	15	23	32	42	53	65	78						
11	4	9	16	24	34	44	55	68	81	96					
12	4	10	17	26	35	46	58	71	85	99	115				
13	4	10	18	27	37	48	60	73	88	103	119	137			
14	4	11	19	28	38	50	63	76	91	106	123	141	160		
15	4	11	20	29	40	52	65	79	94	110	127	145	164	185	
16	4	12	21	31	42	54	67	82	97	114	131	150	169		
17	5	12	21	32	43	56	70	84	100	117	135	154			
18	5	13	22	33	45	58	72	87	103	121	139				
19	5	13	23	34	46	60	74	90	107	124					
20	5	14	24	35	48	62	77	93	110						
21	6	14	25	37	50	64	79	95							
22	6	15	26	38	51	66	82								
23	6	15	27	39	53	68									
24	6	16	28	40	55										
25	6	16	28	42											
26	7	17	29												
27	7	17													
28	7														

TABLE OF WILCOXON TEST ON UNPAIRED SAMPLES

1% Critical points of rank sums

$n_2$ ↓	$n_1$ →	2	3	4	5	6	7	8	9	10	11	12	13	14	15
5					15										
6				10	16	23									
7				10	17	24	32								
8				11	17	25	34	43							
9			6	11	18	26	35	45	56						
10			6	12	19	27	37	47	58	71					
11			6	12	20	28	38	49	61	74	87				
12			7	13	21	30	40	51	63	76	90	106			
13			7	14	22	31	41	53	65	79	93	109	125		
14			7	14	22	32	43	54	67	81	96	112	129	147	
15			8	15	23	33	44	56	70	84	99	115	133	151	171
16			8	15	24	34	46	58	72	86	102	119	137	155	
17			8	16	25	36	47	60	74	89	105	122	140		
18			8	16	26	37	49	62	76	92	108	125			
19	3		9	17	27	38	50	64	78	94	111				
20	3		9	18	28	39	52	66	81	97					
21	3		9	18	29	40	53	68	83						
22	3		10	19	29	42	55	70							
23	3		10	19	30	43	57								
24	3		10	20	31	44									
25	3		11	20	32										
26	3		11	21											
27	4		11												
28	4														

$n_1$  and  $n_2$  are the numbers of cases in the two groups. If the groups are unequal in size,  $n_1$  refers to the smaller.  
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The new Sixth edition of M.N.Ghosh's *Fundamentals of Experimental Pharmacology* is being presented in a new format with a number of additions in the chapters like Pharmacology of receptors, Adrenergic receptors, as well as Opioid and Cannabinoid receptors. There has also been some addition in the chapter dealing with Nitric oxide. New chapters have been introduced like Plant extraction methods, Cell line studies and Nanotechnology contributed by various authors.

### **Excerpts of Book reviews**

The book "*Fundamentals of Experimental Pharmacology*" by Dr. M.N.Ghosh has really been a cornerstone for postgraduate students and researchers engaged in animal experimentation. It has always been useful for postgraduate students in learning and implementing various techniques of experimental pharmacology. Since publication of its first edition in 1971, this book is still considered as a must read and is one of the best amongst many new books published recently.....The recent edition of the book promises to be of an immense help to the postgraduate students and researchers who are genuinely interested in exploring new chemical entities for their further development as a useful new drug.

**Indian Journal of Pharmacology, 44, 430. 2012.**

This revised and revamped 5th ed. by Dr.Ghosh has valuable information for researchers in the field of Pharmacy and Pharmacology....Overall, the author has tried to cover fundamental principles of experimental pharmacology ranging from laboratory animals and methodology to statistical analysis and interpretation of results. Such versatile information could be of immense value especially to post graduates and junior teachers in the field of phamacology and allied sciences.

**Pharma Times,, 44, 34. 2012.**

"*Fundamentals of experimental pharmacology*" includes all the basic topics in details which are in the "must learn" area of the postgraduate student of pharmacology. This knowledge will be required by the medical educators also for guiding the postgraduate students. ....Overall this book is an excellent resource to introduce the postgraduate students of pharmacology to the basics of experimental pharmacology. It will be also of immense help for postgraduate students other than those of pharmacy and pharmacology who want to have a basic knowledge of experimental work on animal

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