CHAPTER - 1

RESUME OF LITRATURE

1. RESUME OF LITERATURE

1.1. Autonomic Nervous System

1.1.1. History

Historically, the adrenergic drugs are both ancient and modern. For the last 5000 years the herb, mahuang has been used in China, among other things, for the treatment of respiratory diseases. About 120 years ago, Vulpain (1856) identified a substance in aqueous adrenal extracts giving a rose-carmine colour on oxidation which, he suggested, might be liberated into the circulation. The effect he was observing was an example of the characteristic reducing property of the catecholamines and as Hagen (1959) points out, Vulpain was unknowingly demonstrating the storage and release of catecholamines for the first time. At about this time, Henle (1865) demonstrated chromaffin tissue histologically with chromium salts which in the light of present knowledge, is a further example of reduction by catecholamines in the tissue stores.

After about 30 years, Oliver and Schafer (1895) found a pressor response when adrenaline (Adr) was injected into an unaesthetized dog, an observation soon to be followed by the identification of Adr as a highly active pressor substance, present in the adrenal medulla (Takamine, 1901). Later it was synthesized by Stolz (1904). Noradrenaline (NA) was also synthetized at this time (Dakin, 1905) although its physiological significance was not appreciated for many years.

The resemblance of the actions of adrenal extracts to those produced by the stimulation of the sympathetic nerves (Langley, 1901) led Elliot (1905) to postulate the chemical mediation of autonomic neural transmission. This hypothesis had to wait 17 years, until the early twenties, before it was conclusively proved (Loewi, 1921).

Although by the thirties, the importance of humoral agents in autonomic nervous activity was firmly established, further progress was retarded by certain contradictory observations showing that Adr failed to mimic all effects of sympathetic nerve stimulation (Cannon and Rosenblueth, 1933). This enigma was not solved until 1947, when both Euler (1946) and Holtz et. al., (1947) noted independently that NA is present in extracts from certain sites in the nervous system, suggesting that it acts as a neural transmitter in mammals. Final proof that this substance is identical with (-) NA was provided a few years later by its detection and estimation in effluent blood from organs subjected to sympathetic stimulation (Main and West, 1950; Oulschoorn and Vogt, 1952).

1.1.2. Introduction to autonomic nervous system

The autonomic nervous system controls tissues (glands, smooth muscle and cardiac muscle) that are not under voluntary control. It consists of two divisions, the sympathetic and parasympathetic, with essentially opposite actions. Thus, if its sympathetic nerve supply is stimulated, the pupil of the eye dilates but stimulation of parasympathetic supply induces pupillary constriction. These facts are well known but some points do require emphasis.

(i) Not all organs receive nerves from both divisions of the autonomic nervous system. Thus, almost all the arterioles in the body are innervated by sympathetic nerves, the tonic activity of which holds the vessels in a state of partial constriction. An increase of sympathetic tone causes vasoconstriction and a relaxation of tone results in vasodilation.

vasodilatation

(ii) Even in organs which do receive a dual innervation, the two divisions of the autonomic systems are not always of equal functional importance. Thus, if the heart rate reflexly accelerates, about 70 per cent of the observed change is due to the withdrawal of parasympathetic tone and the remaining 30 per cent results from an increase of sympathetic tone. Similarly, control of the bladder and the urethra is almost exclusively vested in the parasympathetic nerves.

(iii) Although it is true to say that the autonomic nervous system controls involuntary movements, it must be appreciated that autonomic activity may accompany events initiated in the cerebral cortex. Thus, voluntary motor activity is accompanied by an increased blood flow in the contracting muscles and when muscles are paralysed as a consequence of a cerebral haemorrhage, their blood supply is also disturbed.

(iv) Postganglionic fibres of the sympathetic nervous system bring about their effects by the liberation of NA. Parasympathetic fibres liberate acetylcholine (ACh). ACh is also the transmitter substance at all autonomic ganglia, sympathetic and parasympathetic alike.

1.1.3. Chemicals mediators of autonomic system

The adrenergic nerves, the action of which is mediated by Adr and NA include all the postganglionic sympathetic fibres exclusive of those designated as cholinergic as below. The only known instance of a postganglionic parasympathetic adrenergic fibres is the innervation of the rabbit's stomach.

The cholinergic nerves, the action of which is mediated through ACh include accordingly: (i) all postganglionic parasympathetic fibres (ii) all autonomic (sympathetic as well as parasympathetic preganglionic fibres (iii) the

pre-ganglionic (splanchnic nerve) fibres to adrenal medulla (iv) the sympathetic fibres to sweat glands and certain blood vessels (skin of the face) and (v) the somatic motor nerves to the skeletal muscles.

1.1.4. General effects of autonomic stimulation

Stimulation of the sympathetic nervous system causes dilatation of the pupils, acceleration of the heart, vasoconstriction (particularly in the skin and viscera), the liberation of glucose from glycogen in the liver and muscles, inhibition of intestinal motility and of gastrointestinal secretory activity and constriction of the sphinters. The bronchi dilate, the fur of the animals becomes ruffled and hair of men are erected (giving appearance of goose flesh), the spleen contracts (in those species of animal that possess a contractile splenic capsule) and micturition is inhibited . Stimulation of sympathetic nervous system also evokes the liberation of Adr from adrenal medulla. The Adr powerfully dilates the arterioles in the skeletal muscle and increases cardiac output indicating Adr has different action in different organs.

The activity of the sympathetic nervous system reaches its peak in strenuous exercise and in extreme emotion. This led Cannon and Rosenblueth (1937) to formulate his famous aphorism that sympathetic system prepared the body for 'flight or fight'. It is certainly true that the changes brought about by stimulation of the sympathetic nervous system all facilitate the production of energy, the exchange of respiratory gases and the circulatory adjustments appropriate to intense physical activity. The sympathetic nervous activity is associated with catabolic and parasympathetic with anabolic conditions. The parasympathetic activity will be increased when energy is being taken in, as during the digestion of a meal or conserved as in rest or sleep.

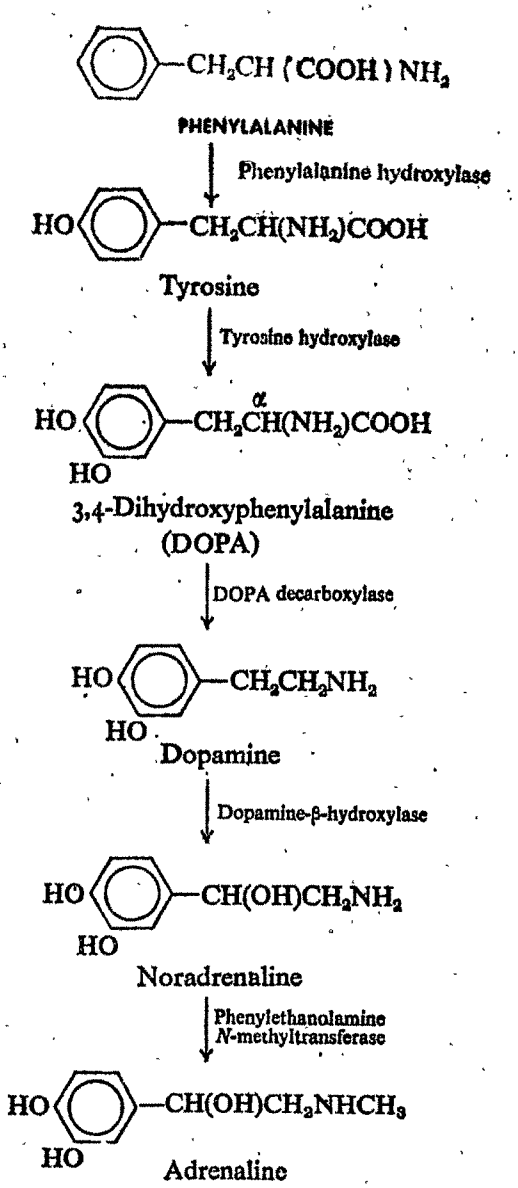
Stimulation of the parasympathetic nervous system induces constriction of the pupils and bronchi, slowing of the heart and an increase in the activity of the digestive system - salivary and gastrointestinal secretions are promoted, the motility of the intestine is increased, the sphincters relax and the gall bladder contracts. The defaection and micturation do not, of course, necessarily, occur in the intact animal, since these functions are to some extent under voluntary control.

Although it is generally supposed that emotional states are associated with activity of the sympathetic nervous system, it should be noted that this demarcation of the physiological function is not so clearcut as is sometimes believed. Some people who receive bad news suffer bradycardia, a fall in blood pressure and collapse; an angry man is likely to be flushed (indicating inhibition of sympathetic activity) and defaecation or micturition sometimes accompany feelings of intense fear of or pleasure. All these events are of parasympathetic rather than sympathetic origin.

1.1.5. Biosynthesis of catecholamines

Many theoretically possible pathways have been proposed for the biosynthesis of NA and Adr; however, that foreshadowed by Blaschko (1939) following the *in vitro* demonstration of L-DOPA decarboxylation (Holtz et al., 1938; Holtz, 1939), has subsequently been shown to be the main route in the mammal (Demis et al., 1956) with phenylalamine as a starting point in the pathway (Fig. 1) includes the following sequences: first para-hydroxylation of phenylalanine to tyrosine by phenylalanine hydroxylase, which is functioning with reduced nicotinamide-adenine dinucleotide ($NADH_2$) as a dihydropteridine reductase (Kaufman, 1959), molecular oxygen (Kaufman, 1964) and reduced pteridine co-factor;

Fig. 1.



Biosynthesis of catecholamines.

Steps in enzymatic synthesis of noradrenaline and adrenaline. (From Blaschko, 1939; Levin et al., 1960).

then metahydroxylation of tyrosine by tyrosine hydroxylase to form dihydroxyphenylalanine (DOPA; Levitt et al., 1965). This step takes place largely in adrenergic neurones of sympathetically innervated tissues and catecholamine storing cells of the adrenal medulla (Sedvall and Kopin, 1967). Tyrosine hydroxylase requires oxygen, Fe^{++} and reduced pteridine probably as a co-enzyme (Brenneman and Kaufman, 1964). DOPA is then decarboxylated by DOPA-decarboxylase in cytoplasmic sap to form dopamine (Blaschko et al., 1955). It has long been known that pyridoxal phosphate is a necessary co-factor for the activation of DOPA-carboxylase (Blaschko et al., 1948). Dopamine enters chromaffin granules (Krishner, 1957) or granulated vesicles in nervous tissue (Potter and Axelrod, 1963) when it is β-hydroxylated by dopamine β-hydroxylase to NA. Dopamine β-hydroxylase requires for its activity agents such as ascorbic acid as an external electron donor, fumaric acid (Levin et al., 1960), ATP (Levin and Kaufman, 1961) Mn^{++} or Co^{++} or Zn^{++}, molecular oxygen and SH-groups (Goldstein et al., 1963). In the adrenal medulla some of the NA thus formed passes into the cytoplasm where it is N-methylated by phenylethanolamine-N-methyl transferase to give rise to Adr (Axelrod, 1962a) which may itself be further N-methylated by the same enzyme to a fourth catecholamine namely N-methyladrenaline. Phenylethanolamine N-methyltransferase has an absolute requirement for S-adenosylmethionine and SH-group but does not appear to be stimulated by Mg^{++} or glutathione (Axelrod, 1962b).

The demonstration of NA synthesis in sympathetic nerves marks a more recent phase in these studies. The conversion of labelled tyrosine or DOPA into dopamine and NA occurs in homogenates of sympathetic nerves or ganglion (Stjarne, 1966). It has been shown to take place centrally in rat and cat brain slices (Masuoka et al., 1963) and in cat brain in vivo (McGeer et al., 1963). Catecholamine synthesis has been demonstrated in animal heart, a rich source of sympathetic nerve (Chidsey et al., 1963; Goldstein and Musacchio, 1963).

Like NA, Adr is found in many tissues of the body in addition to the adrenal medulla although it is not widespread. While its presence in non-adrenal tissue probably stems from uptake of circulating amine synthesized in the adrenal medulla (Axelrod et al., 1959) extirpation of the adrenals, is not followed by complete disappearance of Adr from the urine (Euler, 1946). Thus the evidence points to extra-Adr production and extra-adrenal localization of the responsible enzyme phenylethanolamine-N-methyl transferase (Axelrod, 1962b). The presence of this enzyme has also been noted in brain homogenates (McGeer and McGeer, 1964).

The rate of catecholamine formation in the adrenal medulla and its release into the circulation may be increased by nervous activity (Holland and Schuman, 1956). Sympathetic nerve stimulation also releases NA into the circulation from extra-adrenal sites (Kopin, 1966); however, the discharge of amine from stores is rapidly replenished, suggesting that local synthesis after secretion is linked with nerve stimulation (Anden et al., 1967; Roth et al., 1966).

In general, the NA content of an extra-adrenal tissue is directly related to its sympathetic nerve supply. The vas deferens contains a high density of sympathetic nerves (Flack et al., 1965). It thus possesses a relatively high level of NA, which is unlikely to be supplied from the circulation as the organ has fairly poor blood supply (Kopin et al., 1965). After prolonged preganglionic stimulation of the isolated-hypogastric nerve-vas deferens preparation, a considerable increase in NA formation from exogenous ^{3}H-l-tyrosine has been noted (Roth et al., 1966). In a similar experiment, electrical stimulation of the nerve brought about an enhanced release of ^{14}C-dopamine and NA after prior perfusion of the vas deferens with ^{14}C-DOPA (Austin et al., 1967). These results provide additional evidence of the influence of nervous activity on local NA synthesis. The mechanism of this facilitating action may be associated with the activity of

tyrosine hydroxylase (Levitt et al., 1965), the rate limiting enzyme in catecholamine synthesis. At present, however, this view (Roth et al., 1966) is only speculative; nervous regulation may occur at a latter point than tyrosine hydroxylase in the biosynthesis pathway (Austin et al., 1967).

Catecholamine formation in the mammal takes place in structures belonging to or deriving from the nervous system, largely occuring in adrenal medulla (Goodall and Kirshnar, 1957) extra-medullary chromaffin tissue, heart (Spector et al., 1963), brain (Masuoka et al., 1961), sympathetic nerve and ganglia (Goodall and Kirshner, 1957). Because of their known difficulty in crossing the blood brain barrier (Vogt, 1959), the presence (Carlsson, 1959) and rapid turnover (Carlsson et al., 1960) of dopamine and NA in the brain is indirect evidence for their synthesis in situ; their common precursor, DOPA gains easy access to the brain from the circulation (Carlsson et al., 1958). It thus appears that local synthesis is the main source of endogenous NA in tissues, with uptake of the amine from the circulation making only minor contribution to the maintenance of catecholamine stores (Kopin et al., 1965).

1.1.6. Subcellular distribution of the catecholamine forming enzymes

Differential centrifugation of homogenates of adrenal medulla, post-ganglionic sympathetic nerves and the brain shows the enzymes to be present in cells which normally contain the catecholamines. The enzymes and heavy vesicles in nerves synthesized in the cell body are transported along the axon to the nerve terminals. Tyrosine hydroxylase and DOPA decarboxylase are found largely in the supernatant and so may be cytoplasmic in origin. Dopamine β-hydroxylase activity is present within the catecholamine storage granules of the adrenal medulla, peripheral adrenergic nerves and CNS. Uptake of dopamine into the storage vesicle is a necessary prerequisite to the formation of NA. In the

storage granules some of the enzyme is bound to the inner surface of the membrane and some is in a soluble form, releasable on rupture of the membrane. Nervous stimulation releases the soluble fraction along with the stored catecholamines by exocytosis of the storage granules, hence the appearance of the enzyme in the plasma.

The enzyme methylating NA in the adrenal medulla is found in the supernatant and is presumably in the cytoplasm of the cells. This implies that NA formed in the granules has to be released into the cytoplasm for methylation to Adr, which is then taken up once more into granules.

The present evidence is that the rate-limiting step in the biosynthetic pathway of the catecholamines is the conversion of tyrosine to DOPA; the amount of enzyme is probably small and the tissue concentrations of tyrosine are always sufficient to saturate it. The amounts of the decarboxylase and dopamine β-hydroxylase, however, are present in great excess of normal requirements. Probably for this reason inhibitors of tyrosine hydroxylase are more effective in lowering the content of NA in the tissue than inhibitors of DOPA decarboxylase and dopamine β-oxidase, even when they are given in amounts which cause marked inhibition of the enzymes.

The inhibiting effect of the catecholamines on tyrosine hydroxylase activity may exert a controlling influence on their production in the tissues. Thus, it appears that there may be feedback mechanism whereby catecholamine synthesis is controlled by its rate of secretion and that this control takes place at the step for conversion of tyrosine to DOPA. Prolonged sympathetic stimulation by stress or drugs, however, leads to increased concentrations of the synthesizing enzymes.

1.1.7. Enzymic inactivation of catecholamines

Two enzymes are principally involved, catechol-O-methyl transferase (COMT) and monoamine oxidase (MAO). Since the products of the action of one can then be metabolized further by the other there are several possible pathways of inactivation (Fig. 2).

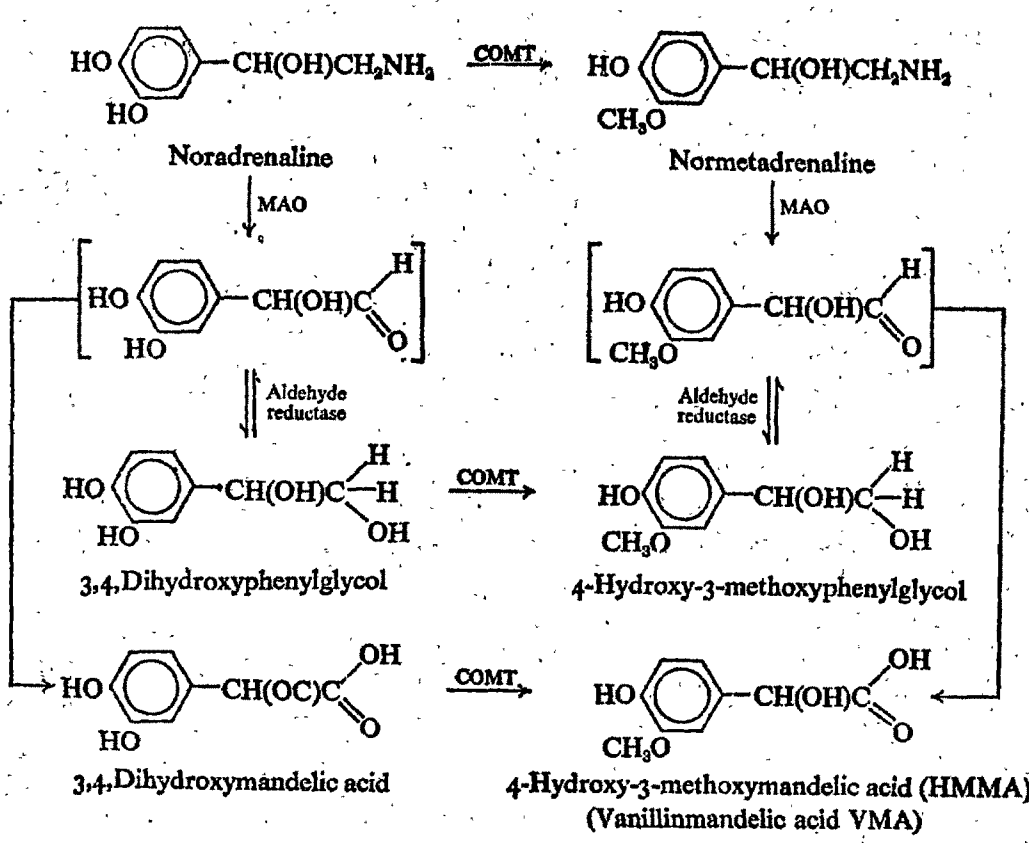
COMT catalyses the introduction of a methyl group into the hydroxy group of the catechol nucleus primarily at the meta - (or 3-) position.

The metadrenaline, normetadrenaline and methoxytyramine are formed from Adr, NA and dopamine respectively. In rare cases depending on the constituent of R, methylation occurs primarily at the 4 position. As with other methyl transferase enzymes, e.g. phenylethanolamine-N-methyltransferase, the methyl group for this reaction comes from 5-adenosylmethionine and studies with purified enzyme from rat liver show that a divalent metal such Mg^{++} is required. Substrate specificity is low and all catedholamines are O-methylated regardless of the substituent at R.

The actions of COMT on the catecholamines and their derivatives can be inhibited both in vitro and in vivo by pyrogallol as well as other catechols. These are competitive inhibitors and are themselves substrates for the enzyme. Other inhibitors are the tropolones, e.g. 4-methyl tropolone, which inhibit by chelating with the divalent metallic ion essential for the activity of the enzyme.

COMT activity is widely distributed in the animal body with high

Fig. 2



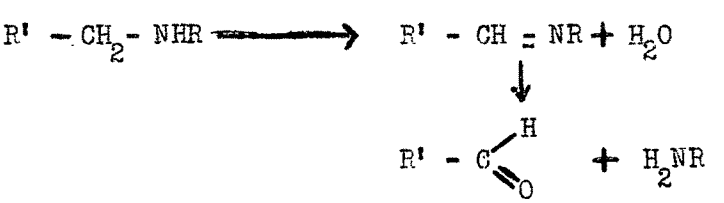
Metabolism of Noradrenaline.

The metabolic pathways for the degradation of noradrenaline. Adrenaline and dopamine are degraded in the same manner. The end products of adrenaline and noradrenaline are the same. The end products for dopamine are 4-hydroxy-3-methoxyphenylethanol and 4-hydroxy-3-methoxyphenylacetic acid (homovanilic acid). (From Passmore and Robson, 1973).

concentrations in the liver and kidney. It is fairly evenly distributed in all the areas of the brain, sympathetic and parasympathetic ganglia and nerves. It is found absent in skeletal muscle or plasma. In sympathetic nerve the amount must be small because surgical sympathactomy with degeneration of nerve has little or no effect on the activity in the peripheral tissues. However, the presence of the enzyme in organs in which the catecholamines act suggest that it acts locally in the metabolism of these substances. The enzyme appears to be located in the cytoplasm of cells.

MAO like COMT is widely distributed and its activity is found in almost every tissue in the body, with high concentrations, in the liver, kidney and intestine. It is also present in all parts of the CNS and in sympathetic nerves.

MAO is a flavoprotein, catalyses oxidative deamination of monoamine such as NA, Adr, dopamine and 3-methoxy derivative arising from the action of COMT. Other amines which also serve as substrates are 5-HT, tryptamine and tyramine. The end product of the reaction is an aldehyde.



In tissue the aldehyde is oxidized by dehydrogenase to the corresponding acid. In case of Adr, NA and their methylated derivatives, the aldehyde can also be reduced to the corresponding alcohol. In brain, dopamine and its methylated derivative give rise mainly to acid metabolites and noradrenaline and its methylated derivatives to alcohol metabolites.

MAO occurs in tissues mainly as an insoluble component of the outer membrane of mitochondria in cells and its activity can be inhibited by a number of substances.

More potent inhibitors have been discovered. These include various hydralazine derivatives such as iproniasid, phenelzine, nialamide and isocarboxazid which produce noncompetitive inhibition of the enzyme. Tranylcypromine and pargyline produce reversible competitive inhibition. Some of these drugs are used in the treatment of depression and hypertension.

1.1.8. Urinary excretion of catecholamines

NA, Adr and dopamine and their metabolites are found in urine. (Table 1) shows some estimates of the amounts found in normal human urine. These figures are collected from different sources and do not refer to the same group of individuals. They indicate the relative orders of magnitude of the different substrates.

The catecholamines, the methoxyamine derivatives and the glycols are excreted partly as conjugates in man, mainly as sulphate esters. In other animals conjugation may predominate.

The rate of excretion of both Adr and NA varies with activity, being lower during sleep or rest in bed than during normal day time routine. Physical stress raises metabolites of both Adr and NA while mental stress raises only of Adr. Insulin raises Adr more while change from the recumbent to the upright position increases output of NA more than Adr.

1.1.9. Uptake of catecholamines

NA and other related amines are known to be transported from the extracellular space across the axonal membrane of adrenergic nerves, a process which for convenience was termed as "uptake"$_1$ by Iversen (1965b). A further mechanism exists to promote the transfer of free catecholamines from the exoplasm into the membrane-bound storage vesicle in adrenergic nerve. Catecholamines are

Table 1. Amounts of catecholamines and metabolites found in human urine (ug/24 hr excretion)*.

Noradrenaline	25-50
Adrenaline	2-5
Normetadrenaline	100-300
Metadrenaline	100-200
4-Hydroxy-3-methoxymandelic acid (HMMA)	2000-4000
4-Hydroxy-3-methoxyphenyl glycol	2000-5000
Dopamine	100-200
Dihydroxyphenylacetic acid (dopac)	2000-4000
Homovanillic acid (HVA)	6000-10000

* (from Passmore & Robson, 1973).

also transported across the membrane and various other postsynaptic cells by another process known as "uptake$_2$" (Iversen, 1965b).

1.1.9.1. Uptake$_1$

Burn (1932) suggested the possibility that exogenous catecholamines might be taken up into the storage sites of peripheral tissues. Raab and Humphreys (1947) and Raab and Gigee (1953, 1955) were the first to demonstrate an increase in the catecholamine content in the cat and the dog hearts after the administration of large doses of Adr and NA in vivo. Nickerson et al., (1950) reported large increases in the Adr content of the rat heart after the administration of Adr. However, Euler (1956) failed to demonstrate an increase in the catecholamine content of various tissues after the administration of smaller doses of catecholamines. C^{14}- Adr was shown to be accumulated in adrenal medulla and several other tissues after intravenous injection (Schayer, 1951; De Schaepdryver and Kirshner, 1961).

The first demonstration of the importance of tissue uptake in the ddisposition of circulating catecholamines was made by Axelrod et al. (1959). After intravenous administration of relatively small doses of H^3-Adr (0-1 mg/kg) to mice, the unchanged hormone disappeared in two phases. In the first five min after injection there was a rapid metabolism of approximately 70% of the injected doses, largely by O-methylation. The remaining 30% however, disappeared only slowly; therafter, detectable amounts of unchanged Adr being present in animals killed several hr after original injection. Other experiments on the fate of H^3- Adr after intravenous infusion in cat showed that unchanged Adr disappeared rapidly from the plasma while at the same time unchaged H^3-Adr accumulated in various peripheral tissues such as heart, spleen, lung and kidney.

Whitby et al. (1961), performed experiments with H^3-NA in cats and mice. It was found that tissue uptake operated to remove the intravenously administered catecholamines from the circulation. The accumulation of NA in tissues was found to be greater than that of Adr. In the mouse, 60% of the injected dose of NA was inactivated by tissue uptake after the injection of a dose of 0.03 mg/kg. Muscholl (1960, 1961) using sensitive bioassay and fluorometric assay technique, demonstrated that an appreciable accumulation of NA in the rat heart was found after the administration of small intravenous doses of NA (0.03 mg/kg). Using fluorometric assay technique, Stromblad and Nickerson (1961) also demonstrated an accumulation of NA and Adr in heart and salivary glands of rats after the administration of amines. These authors suggested that the uptake of catecholamines might represent an important mechanism for the physiological inactivation of catecholamines.

Subsequently, several reports confirmed the ability of various peripheral tissues to accumulate exogenous catecholamines. Increase in catecholamines has been demonstrated in various organs after the administration in vivo in the cat and the rat (Pennefather and Rand, 1960; Harvey and Pennefather, 1962; Crout, 1964). NA uptake has also been demonstrated in isolated hearts or atria of the guinea pig, rabbit, dog and rat (Muscholl, 1960, 1961; Burn and Burn, 1961, Kopin et al., 1962, Trendelenburg and Crout, 1964), perfused cat spleen (Gillespie and Kirpekar, 1963; Blakeley and Brown, 1963) and perfused rabbit kidney (Inouye and Tanaka, 1964). The presence of a blood/brain barrier to catecholamines prevented the study of catecholamine uptake in brain tissue in vivo but studies have been performed with brain slices incubated in vitro (Dengler et al., 1961). Glowinski et al. (1965) and Glowinski and Axelrod (1966) studied the uptake, storage and metabolism of

small doses of H^3-NA in the intact rat brain, administered directly into the lateral ventricle.

1.1.9.1.1. Evidence that uptake$_1$ occurs in sympathetic nerve

In cat it was found that the uptake H^3-NA was greatest in tissues with a rich sympathetic innervation such as the heart. There was an approximate correlation between the amount of H^3-NA accumulated in a given tissue and the richness of the adrenergic innervation, as measured by the endogenous NA content of the tissues (Whitby et al., 1961). In the guinea pig heart and in isolated perfused rat heart there is a significant correlation between the amount of H^3-NA taken up by the tissue and endogenous NA content (Crout, 1964; Iversen, 1967). After superior cervical ganglionectomy, the uptake of catecholamines in the tissues innervated by this ganglion is severely reduced (Hertting et al., 1961; Fischer et al., 1965).

The uptake of H^3-NA is also markedly reduced in various tissues of immunosympathectomized rats and mice in which the development of sympathetic nervous system was suppressed by the administration of nerve growth factor antiserum to new born animals (Iversen, 1965b; Iversen et al., 1966). H^3-NA and H^3-dopamine were perfused in isolated hearts which were pretreated with 6-hydroxydopamine. The accumulation of the amines was greatly reduced in hearts from animals which were chemically sympathectomized by 6-hydroxydopamine (Hellmann et al., 1971). These findings suggest that the uptake of catecholamines occurs mainly in sympathetic nerve terminals. However, Stromblad (1959) reported that the uptake of C^{14}-adrenaline was higher in denervated cat salivary glands than normal glands. Fischer et al. (1965) presented evidence to suggest that the residual uptake in denervated organs represents an accumulation in an extraneuronal site in the tissues. Anden et al. (1963) reported that the uptake

of H^3-NA in the denervated rat salivary gland was not significantly lower than the normal glands if the animals were treated with a combination of inhibitors of the enzymes COMT and MAO.

After injection of H^3-NA, the labelled amine in the heart and pineal gland of the rat was shown to be localized in the postganglionic sympathetic nerve terminals by a combination of radioautography and electron microscopy (Wolfe et al., 1962). Using autoradiographic technique and optical microscopy it has been shown that the accumulated H^3-NA is localized in fine nerve fibres of the brain, spleen and heart of the mouse (Samorajski and Marks, 1962). These results provided first direct evidence for the localization of accumulated NA in the nerve fibres. Hamberger et al. (1964) showed that the administration of NA in large doses (0.5-10 mg/kg) resulted in the reappearance of fluorescent nerve fibres in the rat iris which was previously depleted of NA by treatment with reserpine. These experiments showed that the uptake of NA occured not only at sympathetic nerve terminals but also in preterminal axons and in the postganglion sympathetic nerve cell bodies, in the cervical sympathetic ganglion, suggesting that the uptake of NA may occur at any point on the surface of the postganglionic sympathetic neurone. Malmfors (1965) was able to observe accumulation of NA in the sympathetic nerve plexus of rat iris after the injection of small amounts of NA into the anterior chamber of the eye. Hamberger and Masuoka (1965) demonstrated that the uptake of NA by brain slices incubated with NA <u>in vitro</u> was also localized in the fine nerve fibres in the tissues, which normally contain the endogenous catecholamines. Gillespie and Kirpekar (1965) have combined fluorescent histo-chemical technique with radioautography and demonstrated that exogenous H^3-NA infused into the cat spleen is accumulated in the nerve fibres which contain the endogenous catecholamines.

After the administration of H^3-NA, the labelled amine can be released from the cat spleen by stimulation of the splenic nerve (Hertting and Axelrod, 1961; Fischer and Iversen, 1966), indicating that it is present in the same store as the endogenous catecholamines.

1.1.9.1.2. Kinetics of uptake$_1$

The properties of neuronal uptake process have been studied in a variety of isolated organs or tissue preparations in which external environment can be controlled precisely. Dengler et al. (1961) found that brain or heart slices incubated in a medium containing H^3-NA accumulated the labelled amine to levels upto five times those in the medium. In the isolated rat heart perfused with a medium containing low concentration (10-20 mg/ml) of NA, the concentration of NA accumulated in the tissue rises to levels thirty or forty times those in the perfusing medium (Iversen, 1963; Lindmar and Muscholl, 1964). If it is considered that catecholamine uptake occurs almost entirely into sympathetic nerve fibre in the heart, it is clear that the uptake process has a remarkable ability to concentrate catecholamines. The sympathetic nerves in the heart can only account for a very small proportion of the total weight of the organ. The actual concentration gradient between exogenous NA accumulated in sympathetic nerve terminals and in the external medium must, therefore, be very high, perhaps exceeding 1000:1 (Iversen, 1967).

Dengler et al. (1962) were the first to suggest that uptake of NA was mediated by saturable membrane transport. In these experiments, however, initial rates of uptake at various NA concentrations were not measured. This saturable uptake process has a very high affinity for NA, the apparent ' K_m ' being between 0.2 µM and 1 µM in most rat tissue (Iversen, 1973).

1.1.9.1.3 Stereospecificity for uptake$_1$

The uptake$_1$ is stereochemically selective having an affinity for naturally occuring (-)-NA some five times higher than that for the (+)-enantiomer (Iversen et al., 1971). Kopin and Bridgers (1963) reported that the initial uptake of D-and-L-isomers of NA in the rat heart was the same, although the D-isomer subsequently disappeared more rapidly than the L-isomer. Anden (1964) also found little difference in the accumulation of the stereoisomers in the mouse heart. Crout (1964) reported that there was an equal uptake of the stereoisomers of NA in the guinea pig heart at short time after the injection of D-or L-NA. Maickel et al. (1963) and Bevan and Maickel (1964) achieved a physical separation of the stereoisomers of H^3-NA accumulated in the rat heart after the injection of DL-H^3-NA and showed that there was more than ten times more L-H^3-NA in the tissue a few min after injection, indicating a marked preference for the uptake of L-NA. Iversen (1963) found in isolated rat heart, that the rate of uptake of L-NA was several times more rapid than that of D-NA, when hearts were perfused with low concentrations of the two isomers, the rate of uptake of D-NA was equal or greater than the rate of L-NA, at high perfusion concentrations in membrane transport process; the rate of uptake may be inversely related to the affinity of the substrate for the uptake site if high concentration of substrate are used (Wilbrandt and Rosenberg, 1961). Guinea pig and rabbit tissues appear to lack such stereochemical specificity (Jarrott, 1970).

Apart from NA, a variety of other related amines can act as substrates for neuronal uptake. Adrenaline has an affinity for uptake$_1$ approximately half that of NA in mammalian tissues (Iversen and Whitby, 1962; Axelrod et al., 1959; Whitby et al., 1961). In frog and toad heart, Adr is the preferred substrate for

uptake$_1$ in which Adr represents the predominant naturally occuring catecholamine (Jarrott, 1970). The uptake of adrenaline is also sterochemically in favour of L-isomer (Westfall, 1965; Euler and Lishajko, 1965).

1.1.9.1.4. Ionic requirements

The need of Na^+ for the uptake and storage of NA in sympathetic nerve terminal has been emphasized by a number of workers (Iversen and Kravitz, 1966; Bogdanski and Brodie, 1969; Kirpekar and Wakade, 1968). The requirement of Na^+ is believed to be not unique for the active transport of NA. It was shown that Na^+ plays important role in the cholinergic transmission in ganglion (Birks and MacIntosh, 1961) and transport of glucose across intentinal enithelial cells (Crane, 1962).

The cardiac glycoside ouabain, specially shown to block the Na^+-pump in red blood cells and peripheral adrenergic nerves (Glynn, 1957; Kirpekar and Wakade, 1968) also interferes with the uptake of NA. This supports the view that the uptake of NA is coupled to the active extrusion of Na^+. Furthermore, it was observed that deprivation of external Na^+ causes a rapid loss of intracellular Na^+ due to extrusion. Iversen and Kravitz (1966) demonstrated graded relation of NA uptake in the rat heart to the concentration of Na^+ in the perfusion medium. Both in peripheral tissues and in synaptosome preparations from the central system, the uptake of NA is markedly reduced if Na^+ is absent from the external medium (Bogdanski and Brodie, 1969). Uptake$_1$ also requires the presence of a low concentration of potassium ion (approximately 5 mM) but is inhibited by high concentration of K^+ (above 50 mM). These findings prompted Bogdanski and Brodie (1969) to suggest a model for NA uptake similar to that proposed by other workers for the Na^+ dependent uptake of sugars and amino acids in other tissues. This model

proposes that the uptake of catecholamines depends on the maintenance of the normal inwardly directed sodium concentration gradient by the activity of "sodium pump" and that the affinity with which the carrier sites bind amine is dependent on the relative internal and external concentrations of Na^+ and K^+. White and Keen (1971) found that H^3-NA uptake by synaptosome preparation was not inhibited in the predicted manner by manipulation of the internal Na^+ and K^+ contents and that the inhibition of H^3-NA uptake by cardiac glycosides in synaptosomes did not appear to be correlated with the inhibition of $(Na^+ - K^+)$-ATP ase activity by such drugs.

1.1.9.1.5. Energy requirements

There is a remarkable dependence of NA uptake on the temperature of perfusion fluid (Kirpekar and Wakade, 1968). The recovery of NA in perfusion fluid at 15 °C, 21 °C and 37 °C was 96%, 45% and 34% respectively. The recovery was inversely proportional to the temperature of the perfusion fluid. The rate of uptake of catecholamines approximately doubled for an increase in the temperature of 10 °C and could be inhibited by metabolic poisons such as dinitrophenol and cyanide and by anoxia (Iversen, 1973).

1.1.9.1.6. Inhibitors of uptake$_1$

Burgen and Iversen (1965) and Horn (1973) examined the inhibition of H^3-NA uptake in rat heart and in synaptosomes of rat brain by various phenylethylamine derivatives. The structure/activity relationship for inhibition of uptake$_1$ in the peripheral and central nervous system were essentially similar and can be summarized as follows: (i) affinity for uptake$_1$ sites is decreased by the presence of bulky substituent groups on/terminal nitrogen of the phenylethylamine side/chain, by the presence of methoxy substituents on the ring, and

by the presence of the hydroxyl group on the beta-carbon of the side chain. For the latter compounds affinity for uptake$_1$ sites is greatest for the isomer corresponding to l-NA (ii) Affinity for uptake$_1$ sites is increased by the presence of phenolic hydroxyl groups, particularly in para and meta positions and also by methylation of the alpha-carbon of the side chain. In the latter case affinity is highest for the isomer corresponding to D-amphetamine. It should be pointed out that the structure/activity relations for inhibition of uptake$_1$ by sympathomimetic amines are not identical with the requirements for compounds to be substrate for the uptake process. Thus, for example, amphetamine or beta-phenylethylamine, which lack phenolic hydroxyl groups do not appear to be substrates for uptake$_1$ but are potent competitive inhibitors of H^3-NA-uptake. This suggests that such compounds like competitive enzyme inhibitors are able to bind with high affinity to uptake$_1$ sites in the axonal membrance of sympathetic nerves, but lack the further structural features needed for the inward transport states which normally follow such binding. On the other hand, many sympathomimetic amines inhibit (H^3-NA) uptake because they are competitive substrates for uptake$_1$ (Iversen, 1973).

Apart from the close structural analogues of NA, uptake$_1$ is also inhibited by many other drugs (cocaine, desmethylimipramine, phenoxybenzamine etc.). The most potent inhibitors are found among the derivatives of imipramine, amitriptyline and other tricyclic antidepressants. Imipramine and desmethyl-imipramine are potent inhibitors of uptake$_1$ (Sigg et al., 1963; Iversen, 1965a). Desmethylimipramine is the most potent inhibitor of NA uptake so far described in the isolated rat heart, a concentration approximately 1×10^{-6} M is sufficient to produce a 50% inhibition (Horn et al., 1971). Iversen and Langer (1969) have shown that desmethylimipramine inhibits uptake of NA in rat heart and vas

deferens competitively. Uptake$_1$ is also powerfully inhibited by drugs known predominantly for their other pharmacological activities; such inhibitors include the local anaesthetic drug cocaine; the adrenergic receptor blocking drugs, phenoxybenzamine, chlorpromazine and dichloroisoprenaline, the monoamine oxidase inhibitors, harmine, tranylcypramine and phenelzine; and the adrenergic neurone blocking drugs, bretylium and guanethidine.

Cocaine in addition inhibits the uptake of Adr (Hardman and Mayer, 1965), metaraminol (Carlsson and Waldeck, 1965) and alpha-methyltyramine (Iversen, 1966). In contrast to these reports, it has been found that cocaine fails to inhibit the uptake of catecholamines in the rat uterus (Wurtman et al., 1963) or in sympathetic ganglia (Fischer and Snyder, 1966) suggesting that in these tissues there is either a quantitatively different uptake mechanism or that the drug fails to penetrate the tissues.

Phenoxybenzamine can cause much bigger increase in NA outflow than either cocaine or imipramine (Kirpekar and Puig, 1971). These findings can now be explained although the actions of phenoxybenzamine are complex. Firstly, the drug inhibits uptake$_1$ with a potency comparable to that of cocaine. Secondly, the drug inhibits extraneuronal uptake and metabolism of NA (Lightman and Iversen, 1969), an action which also leads to a greater overflow of unchanged NA (Ianger, 1970). Thirdly, phenoxybenzamine appears to inhibit an "auto inhibition" mechanism by which NA normally acts on presynaptic alpha-receptors to inhibit its own further release during periods of chronic stimulation of sympathetic nerve.

1.1.9.2. Uptake$_2$

Apart from the uptake of catecholamines in the peripheral adrenergic

neurones, the amines are also taken up by a different transport system in various extraneuronal tissues. Although about 95% of the uptake capacity for NA was lost after adrenergic ~~nerve~~ denervation, some uptake did occur; therefore, some tissue component other than the nerve of rat salivary gland is capable of retaining NA (Stromblad and Nickerson, 1961). When Iversen (1965b) perfused isolated rat heart with high concentrations of NA, it was found that the amine was taken up by a saturable process, which could be described by Michaelis-Menten equation and had different properties from that of neuronal uptake. Subsequently, several tissues were shown histochemically or biochemically to be capable of taking up NA, e.g. cardiac muscle of rat (Clarke et al., 1969), smooth muscle of various tissues and rabbit ear artery (Avakian and Gillespie, 1968), cat spleen (Gillespie et al., 1970), vas deferens, bladder, colon, spleen of rabbit, rat, guinea pig and cat (Gillespie and Muir, 1970), cat nictitating membrane (Draskoczy and Trendelenburg, 1970), chick amnion and human umbilical artery (Burnstock et al., 1971), cultured bovine embryonic tracheal cells (Powis, 1973) and collagen (Gillespie et al., 1970). Extraneuronal accumulation of amines was designated as uptake$_2$ to differentiate it from neuronal uptake which was called uptake$_1$ (Iversen, 1965b).

Unlike uptake$_1$, uptake$_2$ involves heterogenous group of cells whose properties with regard to uptake are not identical. For example, the capsular muscle of cat spleen shows no intracellular accumulation, whereas adjacent splenic artery smooth muscle fluoresces brilliantly (Gillespie and Hamilton, 1966). The part of the smooth muscle that accumulated the amine is mainly intracellular with a second, lesser, site on the periphery of the cell in a position corresponding to the basement membrane. The intracellular NA is distributed throughout the cytoplasm with a particular concentration in the nucleus (Gillespie et al., 1970; Burnstock et al., 1971).

Clarke et al., (1969) perfused the rat heart with 10-40 µg/ml of NA and histochemical observation revealed that NA fluorescence was markedly increased in the myocardium and the smooth muscle of the coronary blood vessels in both atrial and ventricular sections. Farnebo (1968) perfused isolated rat heart with NA and alpha-methyl NA and observed marked increase in the amine fluorescence; furthermore, small non-neuronal cells with strong specific green to yellow fluorescence could be found mainly in the connective tissue in the atria.

On exposure to NA, Avakian and Gillespie (1968) observed the development of NA fluorescence in isolated rabbit ear artery; several other tissues, viz. terminal adrenergic fibres, arterial smooth muscle, collagen and elastic tissue could take up the amine though not to a similar extent. All the tissues did not develop fluorescence; nerve bundles of auricular nerve running along side the artery showed little or no NA fluorescence. Smooth muscle and adrenergic nerve terminals resembled one another in that the uptake was intracellular and involved transmembrane transport. Binding to collagen and elastic tissue, in contrast, was not intracellular. The binding to connective tissue was a surface phenomenon involving anionic sites on the fibres or the mucopolysaccharide ground substance but some form of transport mechanism was responsible for the transport of NA across the plasma membrane of the smooth muscle and nerve terminals.

1.1.9.2.1. Species and organ differences and uptake.

There is a remarkable variation in extraneuronal accumulation of NA both between species and between different muscles in a given species. Biochemical estimations of NA retention after cardiac perfusion with 5 µg/ml for 10 min have revealed a marked difference between Wistar rats and C.F.E. rats obtained from Carworth-Europe (Sprague-Dawley derived strain); heart from the former strain accumulated 51% more NA than the latter (Clarke et al., 1969). Among the species,

the ability to accumulate is best developed in the mouse followed by the rabbit, the rat and the guinea/pig in that order. Among the tissues, accumulation is more marked in arterial smooth muscle. There is some correlation between the capacity for uptake in the smooth muscle of an organ and in the smooth muscle of the arteries supplying it. This is particularly well seen in the mouse, where the ability to accumulate NA decreases by more than 10-fold, from the vas deferens through cardiac muscle, bladder, colon to spleen, arterial smooth muscles in these organs show a similar 10-fold range in the ability to concentrate NA, with the same ranking order. There are some exceptions; the smooth muscle of the guinea/pig colon accumulate NA while the capsule and trabecular muscle of spleen do not (Gillespie and Muir, 1970).

1.1.9.2.2. Adrenergic innervation and uptake$_2$

Attempts have been made to relate extraneuronal uptake with adrenergic innervation. It has been suggested that this mechanism of uptake could be useful in smooth muscle with low density of adrenergic innervation enabling the muscle cells to acquire and retain NA in their vicinity (Gillespie and Muir, 1970). This hypothesis was particularly applicable to arterial smooth muscle, where, it has been suggested only the outer shell is influenced by adrenergic nerves, the remainder responding to circulating adrenaline (Gillespie and Rae, 1972). However, investigations on a wider range of tissues showed numerous variations. For example, the muscle of the alimentary canal is poorly innervated, yet with the exception of the guinea/pig and rabbit colon, accumulation is poor, while the mouse vas deferens is densely innervated and its capacity to accumulate is highly developed (Gillespie and Muir, 1970). A second possible link between extraneuronal uptake process and adrenergic innervation was the hypothesis that in densely innervated tissue, the avid uptake by adrenergic neurones would so

deplete the extracellular spaces as to hold the NA concentration below the threshold from smooth muscle uptake. This is not the case, since sympathetic nerve section and degeneration in the cat spleen do not alter the pattern of extraneuronal uptake (Gillespie et al., 1970).

Apart from the innervated tissues, naturally occuring non-innervated tissues, like chick amnion and human umbilical artery are capable of accumulation of NA (Burnstock et al., 1971; Gulati and Shivaramkrishna, 1977). In these non-innervated tissues the threshold of uptake is far lower than that of innervated tissues.

1.1.9.2.3. NA metabolizing enzymes and uptake$_2$

In perfused isolated rat heart, Lightman and Iversen (1969) observed that uptake$_2$ was not a threshold phenomenon, but operates at all perfusion concentrations of catecholamines. At less than 2.5 µg/ml of NA or less than 0.5 µg/ml of Adr, almost all the catecholamines taken up by uptake$_2$ are quickly metabolized. The accumulation of unchanged catecholamines by uptake$_2$ would be expected to occur only when the rate of uptake$_2$ exceeds the maximum rate of metabolism. Thus, if the metabolism of NA is blocked, uptake$_2$ can be demonstrated even during perfusion with low concentrations of NA (0.5 - 1.0 µg/ml). The threshold concentration at which uptake$_2$ becomes apparent as an accumulation of unchanged catecholamines should correspond to the concentration at which uptake$_2$ exceeds the maximum rate of metabolism of catecholamines. Thus, it is not possible to estimate the uptake$_2$ of NA or Adr at lower concentrations in tissues, unless the catabolizing enzymes MAO and COMT are made inactive.

A close relation between extraneuronal accumulation of NA and COMT is striking; while diffusing towards the site of extraneuronal uptake, a considerable

proportion of the NA is O-methylated. Thus block of COMT results in increase in the concentration of the amine at the site of extraneuronal uptake as well as an increased extraneuronal accumulation.

The low concentrations at which the accumulation of NA occurs within the non-innervated smooth muscle (chick amnion and human umbilical artery) corresponds to the threshold for $uptake_2$ after inhibition of both MAO and COMT (Burnstock et al., 1971; Lightman and Iversen, 1969). Burnstock et al. (1972) have shown low activity of COMT and MAO in these tissues. Increased NA uptake has been reported in both rat heart (Eisenfeld et al., 1967) and rat femoral artery (Dolzel, 1966) and in denervated cat nictitating membrane (Draskoczy and Trendelenburg, 1970) following MAO and/or COMT inhibition.

1.1.9.2.4. Kinetics of $uptake_2$

Uptake studies measure the accumulation of NA in a tissue after correcting for the amine in the extracellular fluid. The accumulation can be measured chemically, radiochemically or histochemically; whatever the method, there is an erroneous tendency to equate accumulation with uptake. Properly, uptake should be restricted to the process of transfer of the amine from the extracellular medium onto or into the cell. Whether the accumulation takes place will depend on the fate of the amine transported. If it is metabolized or if there is a diffusional leak back into the medium, the accumulation will occur only when the rate of inward transport (uptake) exceeds the rate of loss (Gillespie, 1973).

Uptake of NA at extraneuronal sites is concentration-dependent; increasing the concentration of NA both increases the rate of accumulation and the equilibrium reached (Iversen, 1965b; Lightman and Iversen, 1969).

1.1.9.2.5. Stereospecificity for uptake$_2$

Neuronal uptake is sterespecific with a preference for the laevo form and a higher affinity for NA than for Adr (Maickel et al., 1963; Iversen, 1963). Extraneuronal uptake is not sensitive to stereoisomers of Adr and NA (Iversen, 1965b; Gillespie, 1968). This uptake system is a low affinity system (higher K_m) but maximum rate of uptake is much greater than that of uptake$_1$ (higher V_{max}) (Iversen, 1965b; Callingham and Burgen, 1966). Uptake$_2$ has higher affinity for Adr than NA; ISO is a still better substrate although this amine is not taken up by neuronal uptake mechanism (Callingham and Burgen, 1966).

1.1.9.2.6. Ionic requirements

The ionic requirements of extraneuronal uptake were studied by Gillespie and Towart (1973). Graded reduction of Na^+ produced graded reduction in uptake, reaching about 50% in the complete absence of Na^+. There are similarities and dissimilarities for ionic requirements from those required for neuronal uptake. The similarities are the partial dependence on Na^+, the partial inhibition by high concentration and the lack of effect of Ca^{++} and Mg^{++}. A striking dissimilarity is the ability of lithium to substitute fully for Na^+. Collagen binding of NA is not affected by the removal of Na^+ (Gillespie and Towart, 1973). In the case of the neuronal uptake of NA, lithium, tris, choline, rubidium, caesium and potassium are all ineffective substitutes of Na^+ (Bogdanski and Brodie, 1969). Lithium has been particularly studied and one study on synaptosomes showed that it could substitute to a small extent for Na^+ (Baldessarini and Yorke, 1970) whereas in extraneuronal uptake, the ability of lithium to substitute completely for Na^+ suggests that the mechanism involved in NA transport into the smooth muscle may be different from that into the nerve.

1.1.9.2.7. Energy requirements

Cooling the smooth muscle to 1-2 °C suppresses uptake. This inhibition is not solely the result of diffusional slowing, since cooling from 37 °C to about 20 °C has little effect; further cooling to 15 °C or lower, greatly reduces the accumulation (Gillespie et al., 1970). This result suggests that uptake is an active process involving the expenditure of energy by the cell. Both uptake and loss of NA from adrenergic storage granule (Stjarne, 1966) and across the axonal membrane (Green and Miller, 1966) are blocked by cold. However, the uptake in chick amnion and human umbilical artery is not affected by low temperature, 1-2 °C (Burnstock et al., 1971). In isolated spleen slices, anoxia combined with glucose deprivation has no effect on NA accumulation in smooth muscle. ATP, which stimulates neuronal uptake (Angelakos, 1964), has no effect on uptake in smooth muscle nor has ouabain in concentrations upto 1 x 10^{-4} g/ml. At 1 x 10^{-3} g/ml ouabain inhibit uptake by about 50% but at this concentration it is difficult to claim this as a specific effect (Hamilton, 1968).

1.1.9.2.8. Inhibitors of uptake$_2$

Uptake$_2$ is inhibited by alpha-adrenoceptor blocking agents, particularly phenoxybenzamine, by normetanephrine and metanephrine (Iversen, 1965b; Iversen and Langer, 1969) and by certain naturally occuring steroid hormones including cholesterol, and by clonidine (Salt, 1972), but is unaffected by cocaine, desipramine or metaraminol which powerfully inhibit uptake$_1$ (Iversen, 1967). The beta-adrenoceptor blocking agent, propranolol in concentrations upto 10^{-4} g/ml is ineffective (Gillespie, 1968; Gillespie et al., 1970).

Among the drugs which block uptake, the alpha-receptor blocking agent, phenoxybenzamine was found to be very effective. This is not entirely a non-specific action, since phenoxybenzamine does not interfere with surface binding of NA on collagen or smooth muscle (Gillespie et al., 1970). It was thus tempting to seek some association between the uptake mechanism and alpha-receptor in particular (Anden et al., 1963; Euler and Lishajko, 1966). Higher concentrations of phenoxybenzamine are needed to block the uptake of NA than to block the responses and in blocking NA uptake, phenoxybenzamine acts competitively and its action is easily reversed (Gillespie et al., 1970). Burgen and Iversen (1965) on the basis of structure-activity relationship came to the conclusion that alpha-and beta-receptors were not related to either $uptake_1$ or $uptake_2$.

Lightman and Iversen (1969) have shown that phenoxybenzamine effectively blocked the extraneuronal uptake of NA and its metabolism. This blocking effect produced by phenoxybenzamine may be competitive, since the effect was reduced at higher concentrations of NA. The estimated ID_{50} of phenoxybenzamine as $uptake_2$ inhibitor was 2.5 μM which suggests that the drug is a potent inhibitor of $uptake_2$ (ID_{50} for metanephrine is 2.9 μM; Burgen and Iversen, 1965). This finding is consistent with that of Langer (1968) that phenoxybenzamine blocks the neuronal and extraneuronal uptake and metabolism of NA.

Iversen et al. (1972) have studied various haloalkylamine derivatives related to phenoxybenzamine as inhibitors of neuronal and extraneuronal uptake in isolated rat heart. Phenoxybenzamine was confirmed to be a potent inhibitor for both $uptake_1$ and $uptake_2$.

The O-methylated products of NA and Adr (metadrenaline, normetadrenaline) are potent inhibitors of $uptake_2$ (Iversen, 1965b; Gillespie et al., 1970;

Burnstock et al., 1971).

Iversen (1965b) found the uptake$_2$ inhibitory activity of normetadrenaline and metadrenaline in isolated rat heart. Subsequently, Burgen and Iversen (1965) found that normetadrenaline had an affinity 60 times that of NA and it had very little or no effect on the neuronal uptake.

Certain steroids are found to be potent inhibitors of extraneuronal uptake of catecholamines. Iversen and Salt (1970) studied the inhibition of uptake$_2$ in isolated rat heart by steroids. Later, Salt (1972) studied in detail the inhibitory action of steroids on uptake$_2$. Steroids produced concentration-dependent inhibition of uptake$_2$ of NA. 17-beta-estradiol, corticosterone, testosterone, deoxycorticosterone and aldrosterone were found to be very potent inhibitors of uptake$_2$ of NA. Studying the structure/activity relationship, Salt (1972) found marked changes in the activity of corticosterone resulting after the addition or removal of hydroxyl radical to the steroid structure. Thus, the 17-beta-hydroxylated derivative of hydrocortisone was completely inactive and successive removal of hydroxyl group from the corticosterone molecule resulted in progressive reduction in the affinity for uptake$_2$ inhibition of NA. Thus, removal of the 11-alpha-hydroxyl group from the corticosterone molecule (deoxycorticosterone) almost halved its affinity and further removal of 21-hydroxyl group from deoxycorticosterone gave a compound (progrestone) with less than one tenth the potency of corticosterone as the uptake$_2$ antagonist.

1.1.9.2.9. Physiological role of uptake$_2$

It is interesting to know whether this uptake operates under physiological concentrations. In rabbit ear artery accumulation of NA could be seen only at concentration of 1 x 10^{-5} g/ml or higher (Avakian and Gillespie,1968).

Above this concentration the slope of the curve relating fluorescence to concentration is steep, when MAO and COMT were left intact, suggesting that 1×10^{-5} g/ml is the true threshold. Such concentrations never occur physiologically in the circulation, but may occur in the vicinity of the nerve endings (Avakian and Gillespie, 1968). In the experiments reported by Gillespie and Kirpekar (1966), short bursts of 200 stimuli to the cat splenic sympathetic nerve released, sufficient NA to give concentrations in the venous blood as high as 5×10^{-7} g/ml in normal cats and 1.2×10^{-6} g/ml in cats treated with phenoxybenzamine. Considering the enormous dilution in extracellular fluid and blood which must occur before NA appears in the venous blood, a concentration at the site of release of 1×10^{-5} g/ml or more seems possible (Avakian and Gillespie, 1968). In the hope that uptake of NA in the vicinity of nerve endings could be demonstrated during high frequency stimulation, lengths of rabbit ear artery were removed during stimulation at 30 Hz and immediately frozen. Examination of the artery treated with Falck technique showed some local diffusion of the transmitter with fluorescence of adjacent structures (Avakian and Gillespie, 1968). Langer (1970) has provided further evidence that the extraneuronal uptake and metabolism of NA accounts for a substantial proportion of NA released at adrenergic synapse in the cat nictitating membrane. A similar local diffusion with nerve stimulation has been reported by others (Gillespie, 1968) reinforcing the likelihood that extraneuronal uptake can occur under physiological conditions.

1.2. Receptors

1.2.1. Introduction to receptors

Pharmacologically active substances may be roughly divided into two groups: (i) those compounds whose biological activity can be correlated in terms

of some general physical property and is only indirectly dependent on chemical structure, for example, volatile anaesthetics (Ferguson, 1939); (ii) those compounds whose activity is dependent on chemical structure and where minor alterations in this structure can have a profound effect on activity. It is in the attempt to rationalize the behaviour of this latter class of compound that the idea of a "receptor" is most frequently introduced. The concept of a receptor proves to be remarkably elusive, considering the central position it occupies in the theory of pharmacology; and any attempt at a rigorous definition is probably fruitless (Schild, 1962). A receptor will be considered as the component of a cell with which a drug combines to initiate a response. The vagueness of this definition reflects our lack of precise knowledge about receptors. The idea of receptors originated long before it was generally accepted that matter consists of atoms and molecules.

Paracelsus around 1500 A.D. stated that drugs should contain 'Spicula' (Barbed hooks) with which they could become fixed to the organism and so produce an effect. Langley in the year 1878 first suggested the existence of a "receptive substance" to explain the actions of curare and nicotine and this idea has gained common currency for almost as long as pharmacology has existed as a distinct body of knowledge; it is the keystone of almost all discussions of structure-activity relationships and of the quantitative description of the effects of drugs; yet, in so far as it is not yet possible to write a recognizable chemical structure for one receptor, it could be said that no more is known about receptors now than in the days of Langley. It is however, not difficult to find reasons for this apparent lack of progress. The ability to differentiate sharply between different chemical substances is one of the most fundamental properties of living systems and the central problem of pharmacology - why an organ responds in a certain way to a particular drug - is only one part of this larger question.

Ehrlich was also the father of chemotherapy and it was his work in this field which led him to the conclusion that specialized regions of the cell, the receptors, were vital in the organism's function in as much as they were the means by which it attached itself to nutrient molecules. Substances which poisoned the cell did so, Ehrlich surmised, because they could attach themselves to the receptors thereby depriving the organism of its means of obtaining nourishment. The toxicity of the substance would be considerably increased if, as well as being able to attach itself to the cell, it possessed another chemical grouping (the toxophoric group) capable of actively injuring the cell. Ehrlich first published these ideas in 1900. In later years, he extended them so as to embrace the more general case of drug action on excitable tissues. Like Langley, Ehrlich took as his model the antagonism of atropine and pilocarpine; he assumed that both compounds would, by means of 'anchoring groups' attach themselves to the receptors of the tissue cells. The fact that atropine, in contrast to pilocarpine, has no excitatory action on the tissues, was seen as being due to the different activities of the active groups (analogous to the toxophoric groups) in the two substances. Ehrlich's views are epitomized in his phrase 'Corpora non agunt nisi fixata' (substances do not act unless fixed) - the only Latin tag which finds a place in modern text books of pharmacology and itself derived from the chemists' rather more famous 'Corpora non agunt nisi liquida'. Ehrlich linked the relationship of drugs and their receptors to a lock and its matching key, just as Fischer (1894) had pictured enzymes and their substrates.

Fischer (1894) coined the graphic image of the lock and key to explain enzyme specificity and this idea, writ large as 'molecular complementarity' has offered a satisfying general explanation of the profound alterations in biological activity that can often follow minor alterations in the structure of a drug. Briefly, it is suggested that the receptor has complementary

structure to the drug and that, owing to the weakness of the forces binding the drug to the receptor, a very close fit between the two is needed for firm binding. Owing to the cooperative and complementary nature of these binding forces any deviation from the optimal structure, for example, by the substitution of a bulky group for a small one, by rearranging the constituent groups of a molecule in space by inversion at an asymmetric centre, or by altering the direction of a dipole within the molecule can cause a profound alteration in the stability of the drug-receptor complex. This explanation is probably basically correct and indeed it is difficult to conceive of any other explanation for the great changes in biological activity that often follow inversion of an asymmetric centre in a molecule (Beckett, 1959).

The receptor theory of drug action implies that the pharmacological properties of a compound are dependent not only on the nature and properties of the constituent groups within the molecule but also in the way in which these groups are distributed in space. This follows from the idea that the receptor is a discreet, spatially organized structure and that maximum activation of the receptors only occurs when there is close apposition between the drug and the receptors. The size and shape of groups in a molecule and their spatial arrangements has been emphasized recently by Ariens and Simonis (1964).

The first attempt to incorporate the receptor concept into some quantitative generalizations was made by Clark in 1937. Clark assumed that the response of a tissue was dependent on the number of receptors which were occupied by the drug and that the interaction between drug and receptor was a simple unimolecular reversible process. Ehrlich (1900) and Clark (1937) had clearly recognized attachment to a receptor is a necessary but not a sufficient

prerequisite for pharmacological activity. A compound may become attached to a receptor but it will elicit no visible response unless it is also capable of setting into motion the train of events which culminates in an overt response of the tissue. The ability to do this is referred to as the efficacy (Stephenson, 1956) or the intrinsic activity (Ariens, 1954; van Rossum and Ariens, 1962). The more recent definition of intrinsic activity makes it virtually identical with efficacy and the two terms may be used interchangeably. It should be noted that a drug with a low affinity but a high intrinsic activity can produce the same pharmacological response as one with a high affinity but a low intrinsic activity.

At the present state of knowledge, any discussion of drug-receptor interactions must be highly speculative: something is known of the structure of the drug; partically nothing is known about the equally important second component of the reaction- the receptor. An indication of the kind of reaction that may be occuring can be made and it may be asserted that the action of drugs on cellular systems is understandable in principle in terms of forces that are already known. Any more explicit statements concerning the receptor must await the completion of the formidable task of the isolation and complete structure determination of the receptor substance.

Although receptors for most drugs have yet to be identified, there is little doubt that drug-cell combinations obeying mass law kinetics are involved in drug actions. The many discreet relationships between chemical structure and biological activity and the competitive interaction of chemically similar drugs are difficult to explain except in these terms. Receptor groups like the active centres of enzymes are thought to be carboxyl, amine, sulfhydryl, phosphate and similar reactive groups spatially oriented in a pattern complementary to that of the drugs with which they react. The binding of drug to receptor is thought to be

accomplished mainly by ionic and other relatively weak, reversible bonds. Occasionally, firm covalent bonds are involved and the drug effect is only very slowly reversible.

Receptor theory has often been extended to explain the shape of the dose-effect relationship and to interpret the interaction of drugs that act at other than a common receptor site. For these applications, it is necessary to make some assumptions about the relationship between drug-receptor interaction and intensity of drug effect. In the classical receptor theory developed by Clark (1937), it was assumed that drug effect is proportional to the fraction of receptors occupied by drug and that maximal effect results when all receptors are occupied. Neither of these assumptions is likely and subsequent modifications of the occupation theory have assumed other relationships between receptor occupation and drug effect and have permitted the possibility that maximal effect may be achieved when only a portion of receptors is occupied. The latter concept is described as that of spare receptors. It has also been proposed that drug effect may be a function not of receptor occupation but of the rate of drug-receptor combination. This rate theory is attractive because it relates drug efficacy to the rate of dissociation of the drug-receptor complex and explains certain other aspects of the time course of drug effect. At the present time, it is impossible to choose between the various theories of drug action and their major value is that of providing a conceptual framework for designing future experiments. It deserves emphasis, however, that mechanism of drug action is defined not by an equation relating dose and effect or describing the pattern of drug interaction, but only by identifying the role of drug receptors in normal cellular function and by characterizing the action-effects sequence. It is not unreasonable to look to enzymes and other active macro-

molecules for clues to the structure and activity of drug receptors. Some drug receptors are enzymes but others are not. Acetylcholine must clearly have close structural affinities with active groups on both cholinesterase and receptor molecules, but it is difficult to sustain the view that enzyme and receptor are identical.

Recent studies of enzyme structure suggest that substrates are capable of inducing conformational changes in enzyme molecules and a similar change may well take place when a drug moleculemeets its receptor. This has led Mautner (1967) to suggest that two specialized regions of the drug molecule should be distinguished. One enables the compound to become attached to the receptor molecule and it is responsible for the drug's affinity. The other portion of the molecule which induces a configurational change in the receptor (and hence initiates the pharmacological response) determines the drug's intrinsic activity. An antagonist is incapable of bringing about this change (it has zero intrinsic activity) and a partial antagonist has only a limited ability to do so. It will be realized that this hypothesis, the best we have, echoes that of Ehrlich which was propounded so long ago. This is at once a tribute to the latter's prescience and a confession of our known inability to penetrate to the heart of the structural unit of pharmacological activity.

There can be little doubt that receptors exist as actual entities. If suitable tissue is exposed to a radioactively labelled drug, the sites at which the drug is bound can be revealed by autoradiography. Using this technique, Waser (1966) demonstrated that tubocurarine, a competitive antagonist of acetylcholine, accumulated at the end-plates of the muscle fibres in the mouse diaphragm. He calculated that 1.4×10^6 molecules of tubocurarine were bound at each end-plate. This presumably reflects the number of acetylcholine receptors in each muscle cell: it is quite surprisingly close to the figure (1.6×10^5

receptor molecules per cell) calculated for the longitudinal muscle of guinea-pig ileum by Paton and Rang (1965).

1.2.2. Types of receptors

1.2.2.1. Cholinergic receptors

It was mentioned above that ACh is the only transmitter for the somatic and the ganglionic innervation in the entire autonomic system and for the parasympathetic division at the postganglionic level. There is a clear-cut difference between the receptors of each of these regions, which was demonstrated long ago with pharmacological agents. This has been the basis for the recognition of muscarinic and nicotinic types of cholinergic receptors.

The term muscarinic receptor derives from the fact that the physiological effects of ACh can be simulated with muscarine, a drug obtained from the fungus Amanita muscaria. Such an effect can be observed in smooth muscles, exocrine glands and cardiac muscle and can be blocked by atropine but not by d-tubocurarine.

The term nicotinic receptor derives from the fact that the effects of ACh can be mimicked by nicotine, an alkaloid from Nicotiana tabacum. Both the somatic innervation and those of the preganglionic fibres of the autonomic system are nicotinic. The myoneural junction is blocked by d-tubocurarine but not with atropine, whereas the ganglionic synapses are blocked with hexamethonium and triethylammonium.

A number of drugs of different potencies can act on nicotinic and muscarinic receptors. Many quaternary and tertiary ammonium salts function as cholinomimetics, the former being the most active.

1.2.2.2. Adrenoceptors

In adrenoceptors, as in cholinergic receptors, different types can be recognized. In 1948, Ahlquist differentiated the so-called alpha-and beta-adrenoceptors. One of the criteria used to differentiate receptors was the relative potency of different catecholamines to produce the same physiological effect. For example, in the constriction of different vascular areas (uterine contraction in the rabbit, contraction in the cat nictitating membrane), there is the followirg gradient of potency: Adr > NA > phenylephrine > ISO . In vasodilation, at inhibition of uterine contraction and change in the inotropic and chronotropic effects on the heart, the order ISO > Adr > NA > phenylephrine was observed.

The classification of Ahlquist (1948) was later complemented by the criterion of selective action of blocking agents for alpha and beta receptors. This criterion elucidates only the most evident differences. Adrenoceptors may vary from tissue to tissue and from species to species, however; and several subclasses have been poctulated.

The development of special blocking agents permitted a better definition of α-and β-receptors. Among the α-blocking agents, in addition to the ergot alkaloids, we have the reversible competitor phentolamine and those agents that may compete in an irreversible manner, such as the β-haloalkylamines. None of these drugs blocked the stimulating effects of catecholamines on the heart. Powell and Slater (1958) introduced a series of drugs that have this property and since then such β-blocking agents as dichloroisoprenaline, pronethalol and propranolol have become widely used. Furchgott (1967), using a pharmacological approach, recognized the α-nature of receptors in several tissues of the rabbit. Two catecholamines with an α-effect, NA and dopamine (DA) act on a common receptor of the pulmorary artery of the dog (Kauman and Ochoa, 1970).

The situation regarding β-receptors is more complex; receptors of this type but having special characteristics, were found in tissues of the guinea pig and rabbit. The existence of atleast three subclasses of β-receptors was postulated (Furchgott, 1967). In the heart the β-receptors that mediate the positive inotropism seem to be identical to those determining positive chronotropism (Blinks, 1967). Land et al., (1967) in experiments based on the selective action of the new β-blocking agents, differentiated between β_1 and β_2 receptors. β_1-receptors are found in heart, β_2-receptors in the bronchi. Their studies involve, in each case, the determination of pharmacological constants such as the apparent dissociation constant (Furchgott, 1967) or the pA_2 of Arunlakshana and Schild (1959). As in the case of the cholinergic receptors, most of our knowledge about the possible structure of adrenoceptors is indirect and is derived from studies on the conformation of the agonists in solution (George et al., 1971).

We have already seen that most tissues innervated by the sympathetic system, have both α-and β-receptors. This complicates the biochemical analysis that eventually may lead to the isolation of the receptor macromolecules. An exception to this rule seems to be the heart ventricle of the cat that contains only β-receptors (Nickerson and Chan, 1961), and, for this reason, it was a very appropriate tissue in which to try to isolate the β-receptor.

Pharmacologically, these cardiac receptors react to both L-NA and L-ISO (Blinks, 1967) and are supposed to be localized in the membrane of heart muscle cells, a suggestion first put forward by Clark (1926).

1.2.2.3. Receptors for amino acids

For more than two decades it has been known that the amino acids, glutamic acid and gamma-amino butaric acid (GABA), occur in high concentration in brain of

vertebrates, and that when applied at the surface of certain neurons, they affect electrical activity. The possible transmitter role of these two amino acids, as well as, more recently, that of glycine, has been reported (Curtis and Johnston, 1974).

1.2.2.3.1. GABA receptors

In the case of GABA, although it was first discovered in the vertebrate brain (Awapara et al., 1950), its function as an inhibitory transmitter was first demonstrated in the crustacean neuromuscular junction. In fact, when GABA was tested for biological activity on the crustacean stretch receptor, it was found that it reproduced the effects of the stimulation of the inhibitory axons (Bazemore et al., 1957). Furthermore, it was shown that picrotoxin could block the response of the muscle both to nerve stimulation and the effect of GABA (Robbins and Vander Kloot , 1958).

From the neurochemical point of view, it was demonstrated that the inhibitory axons innervating crustacean muscle contained as much as 0.1 M (Moles/litre of axoplasm) of GABA, whereas in the excitatory nerves, the concentration was less than 0.001 M (Kravitz and Potter, 1965). Furthermore, the glutamic acid-decarboxylase, i.e. the enzyme that synthesizes GABA from glutamic acid, is only found in inhibitory axons (Hall et al., 1970). The inhibitory role of GABA in the crustacean neuromuscular junction was further supported by the demonstration that this amino acid is released after stimulation of the inhibitory nerves and can be collected in the superfusate (Otsuka et al., 1966).

The similarities between the stimulation of inhibitory nerves and the action of GABA were confirmed by studies on the membrane conductance of

crustacean muscle fibres. Using intracellular recording Boistel and Fatt (1958) observed that both treatments increased chloride conductance and Takeuchi and Takeuchi (1965) found that both treatments drove the membrane potential toward the same ionic equilibrium potential, determined by the increase in chloride conductance. The identical action of GABA and of the inhibitory nerve was further confirmed by the observations that picrotoxin and γ-guanidinopropionic acid (Dudel, 1965; Takeuchi and Takeuchi, 1969) selectively blocked the response of the muscle receptor and increase in chloride conductance. The mechanisms by which GABA is inactivated, after its release from the nerve ending, has been investigated by Kravitz and Potter (1965). Such mechanisms may involve a rapid enzymatic degradation by GABA-amino-transferase or a reuptake, as in the case of NA. The latter seems to be possible since there is a selective accumulation of GABA from the extracellular fluid which is Na-dependent (Iversen and Kravitz, 1966). Using radioactive GABA, Orkand and Kravitz (1971) attempted to localize the sites of accumulation of this amino acid. The autoradiographic label was mainly found in the cytoplasm of connective and supportive cells, it was less marked over muscle fibres. There was practically no accumulation of ^{3}H-GABA over the neuronal structures.

Pharmacologically, the study of receptors for GABA has been mainly carried out in the CNS of vertebrates. Here the problem is complicated by the fact that glycine is also an inhibitory transmitter. This problem has been partially ~~s~~solved by the demonstration that strychnine selectively antagonizes the effect of glycine (Curtis et al., 1968). The strychnine resistant inhibition has been found to be blocked by picrotoxin (Galindo; 1969; Bruggencate and Engberg, 1971) and the alkaloid bicuculline (Curtis et al.,1970).

1.2.2.3.2. Glutamate receptors

Although glutamate is well recognized as a leading candidate for excitatory transmitter in primary afferent neurons conveying somatic and visceral sensory information into the CNS of vertebates, it is in the neuromuscular junction of arthropods (Florey, 1967) that the transmitter function of this amino acid has been best studied.

There is good evidence that, in both crustacean and insects, motoneurons produce glutamate as the transmitter substance (Florey, 1967). Of a large number of compounds tested, only glutamate and to a lesser degree, aspartate were effective in producing contraction of the muscle in crustacean (Robbins, 1959). The excitatory effect of glutamate has been best studied in the neuromuscular junction of insects (Usherwood and Machili, 1966).

In the retractor unguis muscle of the locust, the striated muscle fibres are multi-innervated by T-shaped nerve terminals from motoneurons that exert an excitatory action. L-Glutamate depolarizes insect muscles at concentrations of 10^{-7} g/ml, and is more effective when applied electrophoretically in brief pulses. D-Glutamate produces no response at concentrations below 10^{-3} g/ml, and l-aspartate or d-l-homocysteate have little effect. It is interesting that the sensitivity to L-glutamate is localized at the junctional surface and that upon denervation, it extends to the entire surface of the muscle fibre.

By electron-microscope autoradiography, an attempt was made to localize the sites of ^{3}H-glutamate uptake. As in the case of ^{3}H-GABA, in the crustacean neuromuscular junction, the ^{3}H-glutamate was found mainly in sheath cell surrounding the nerves to the muscle (Faeder and Salpeter, 1970). The uptake of ^{3}H-glutamate was found to be greater in the region close to the junction and to be enhanced by electrical stimulation.

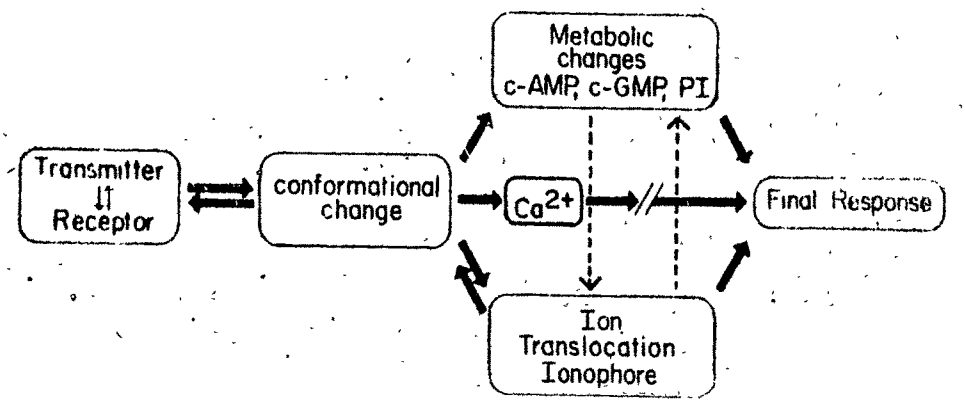
1.2.2.4. Synaptic receptors

As discussed, it seems probable that synaptic and other pharmacological receptors are genetically determined macromolecules, which are localized within the structure of the cell membrane and which have specific sites that recognize endogenous or exogenous ligands. It is generally assumed that the ligand-receptor interaction triggers a conformational change in the macromolecule, which initiates the series of changes in the membrane, i.e. translocation of ions, displacement of Ca^{++}, and changes in membrane potential , which eventually lead to a response of the entire cell, i.e. contraction, secretion, metabolic response (Fig. 3). This definition excludes as receptors those macromolecules that act as receptors, binding nonspecifically to the ligand (Furchgott, 1964) and the enzymes that may act to modify ligand concentration, i.e. AChE, monoamine oxidase (MAO) and so on.

In fact, in none of these cases does the interaction with the ligand induce a physiological response. In spite of the fact that the action of certain enzymes may change the response by affecting the level of neurotransmitter at the receptor site, they only induce a modulation of the response but not a true receptor response.

In his book on Neurotransmitter-Receptor Interaction, Triggle (1971) mentions that 'there is inadequate information about the neurotransmitter receptors, how they are integrated in the cell membrane, how the events initiated at these receptors are coupled to ionic, potential, biochemical and mechanical changes '. later he states that " it is distinctly possible that this picture will change dramatically over the next few years for there is a rapidly developing awareness of the regulatory significance of cell membrane processes".

Fig. 3.



The diagram shows the primary interaction between the neurotransmitter and the receptor that takes place at the cell membrane. This is followed by a series of intermediate steps, which finally produce a physiological response, such as the contraction or relaxation of a muscle or the secretion of a gland. The figure also emphasizes that the primary interaction produces a conformational changes, which, in turn, may induce the displacement of ionic calcium, metabolic changes (i.e. cyclic AMP and GMP, phosphoinositides) and conductance changes. The latter by the translocation of ions may give rise to changes in membrane potential. (From Eduardo DeRobertis, 1975).

This view is correct since this is an era of dramatic change in which receptors may be isolated and their molecular properties studied by means of biochemical and biophysical techniques (De Robertis, 1971).

1.2.2.5. Central nervous system receptors

The study of the central nervous system of vertebrates by modern cytochemical techniques (De Robertis, 1967) has revealed that there are different types of neurons with different biochemical characteristics and that in general terms each type produces a unique neurotransmitter. Furthermore, pharmacological studies using microelectrophoretic injections of different drugs have demonstrated that these transmitters have different chemical reactivities, implying the existence of different types of receptors at the cell surface of a single neuron. In addition to ACh and NA such biogenic amines as 5-hydroxytryptamine (5-HT), dopamine and the amino acids such as glutamic acid, GABA and glycine may act as CNS transmitters. Thus, specific receptors for these and other possible transmitter substances should be present in the neurons of the CNS.

Since the beginning of research on the ultrastructure of central synapsis, one of the important goals was to identify morphologically the organelles involved in synaptic transmissions. The finding that presynaptic endings contained special synaptic vesicles (De Robertis and Bennett, 1954) suggested that the transmitter was stored in these vesicles, a fact that was later proved by the isolation of this vesicular component (De Robertis et al., 1963).

The above observations did not suggest the exact locus at which the vesicle discharged to release the transmitter, thus initiating the process of

synaptic transmission. Regions of increased thickness and electron density were first observed by De Robertis (1955) in the cochlear nucleus. Palay (1958) made similar observations and described these regions as 'active' points of the synapse. The synaptic cleft was found to be wider than the usual extracellular space and a dense subsynaptic material was observed at the site of the active points (De Robertis, 1959). Differences in these junctional thickenings were used by Gray (1959) to differentiate different types of synapses in the CNS. A system of intersynaptic filaments of macromolecular size joining the two synaptic membranes across the cleft and a subsynaptic web of the fine filaments were also observed (De Robertis et al., 1961). These studies led to the recognition, as a special structure, of the so-called junctional complex, which includes the two synaptic membranes, the cleft with the intersynaptic filaments and the subsynaptic web.

An interest in the isolation and characterization in central receptors started as a consequence of the work on cell fractionation of the cerebral cortex and other areas of gray matter. Cell fractionation of the CNS involves homogenization of the tissue, followed by differential and gradient centrifugation of the primary fractions obtained. Electron micrographs of the different nerve-ending fractions isolated revealed that these structures had attached to them portions of the subsynaptic membrane where the receptor was thought to be present (De Robertis et. al., 1962).

1.2.2.6. Hormone receptors

What awakened endocrinologists to the reality of hormonal receptors was the discovery that tissues that had been identified in physiological studies as likely target tissues for oestrogens such as the uterus and vagina, contained binding

sites to which ostrogens became bound with high specificity and affinity (Glascock and Hoekstra, 1959). In presumed nontarget tissues, such as liver and muscle, the binding sites appeared to be absent. Subsequently, by a variety of techniques, comparable hormone-specific binding sites were demonstrated in target tissues for mineralocorticoids (Edelman et al., 1963), glucocorticoids (Schaumburg and Bogesen, 1968;Munck and Johnsen, 1968), androgens (Bruchovsky and Wilson, 1968) and progestins (Wiest and Rao, 1971). All of these binding sites were eventually shown to be associated with protein molecules.

Such protein molecules clearly fulfill two of the essential requirements for receptors: they are present in target tissues and are capable of "receiving" the hormone message through a specific molecular interaction. A third requirement, that they should be able to translate the hormone message into a hormone effect, has so far not been satisfied unequivocally by any of the current candidates for receptors.

A general definition of a receptor R is that it is a molecule or group of molecules that interacts with a hormone H through specific binding sites to form a hormone-receptor complex HR, which, in turn , mediates the events in the cell that ultimately are amplified into physiologically recognizable effects of the hormone. As with any definition, this one is subject to interpretation in light of contemporary views. At present, the prevailing view is that steroid hormone receptors are macromolecules that interact with hormones to form hormone receptor complexes with high affinity for nuclear acceptor sites. Not long ago, however, it was reasonable to think that receptors were carriers, the purpose of which was to carry the hormones to the nucleus, where they were presumably transferred to other receptors. In pharmacological usage, receptor-binding sites may be active sites of enzymes, the drug being a substrate or cofactor (Ariens, 1964). Such a role was contemplated for steroid hormone receptors in the fashionable theories of hormones as coenzymes (Hechter and Halkerston, 1964).

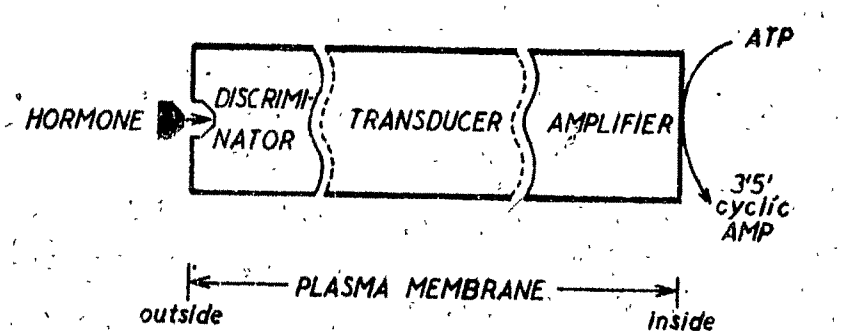
1.2.3. Relation of receptors with cyclic AMP and cyclic GMP

1.2.3.1. β-Receptor and adenylate cyclase

A point of extreme interest is the possible relationship between the β-receptor and adenylate cyclase. Sutherland and Rall (1960) first described the stimulation of liver adenyl cyclase by NA. Since then a large number of hormone actions have been shown to be mediated by the stimulation of this enzyme in various target tissues (Rall and Gilman, 1970). This hormonal effect is tissue specific; in fact the adrenal cyclase is stimulated by ACTH, that of the thyroid by TSH and the cardiac cyclase by Adr. This implies that in each case a different "receptor" should be used to recognize the hormone structure that will activate the cyclase system.

The study of the hormone-receptor interaction in vivo permits a better analysis of the specific control mechanisms than that of the intact cell or organism. In liver, as well as in fat cells, the adenylate cyclase system is localized in the plasma membrane (Rodbell et al., 1971); similarly, in brain it is concentrated in the nerve-ending membrane (De Robertis et al., 1967). In these and in other cells, it has been demonstrated that the enzyme active site, which synthesized 3'-5'-cyclic AMP from ATP, is situated at the inner surface of the plasma membrane. Rodbell et al., (1971) have theoretically represented the adenylate cyclase system as an information-transfer system in which there are three components. At the cell surface there is a receptor (the so-called discriminator), which specifically recognizes the hormone. The information gained is coupled by means of a transducer to the amplifier or cyclase itself (Fig. 4). By way of this information-transfer system, the low-level signal offered by the hormones (which may act at concentrations of 10^{-9} to 10^{-12} M) may be amplified one thousand times by the production of the 3'-5'-cyclic AMP. In the case of fat cell ghosts, the enzyme may react to several hormonal influences. This could mean that several different receptors may feed into a single type of

Fig. 4.



Representation of the adenylate cyclase system as an information-transfer system. Systems are present in plasma membrane of target cells and consist of a component (discriminator) that specifically recognizes a hormone. Information gained through the complex of hormone-discriminator is coupled (tranducer) by undetermined physical forces to the enzyme adenylate cyclase (amplifier), which, in the presence of an energy source (ATP), amplifies and converts the hormone signal to a new signal (3'-5'-cyclic AMP) for use by the various information-transfer systems in the target cell. (From Robell et al., 1971).

amplifier system. According to Rodbell et al. (1971), the binding of the hormone appears to involve a lipoprotein of the plasma membrane.

Studies on metabolic effects of catecholamines have shown that they are mediated by the β-adreneceptor and the adenylate cyclase system (Robinson et al., 1967). Since this enzyme system is activated in a similar way as the β-receptor and blocked with β-adrenoceptor blockers (i.e. propanolol), it was postulated that either the enzyme and the receptor were identical or that, in the enzyme, there were two subunits, one acting as a receptor to the external stimulus, the other being involved in the synthesis of cyclic 3'-5'-AMP.

In the heart, Murad et al. (1962) demonstrated that catecholamines increase adenylate cyclase activity in particulate preparations of dog myocardium. Similar findings were obtained in the human being (Levey and Epstein, 1969) and in the rat (Murad and Vaughan, 1969). Another similarity was that the following potency series of agonists i.e. ISO > NA or Adr > Dopamine > DOPA were found to activate the β-receptor and adenylate cyclase (Lefkowitz and Haber, 1971). The general conclusion was that, in myocardium, the positive inotropic effect produced by catecholamines is mediated by the activation of this enzyme system. The resulting increase in the level of cyclic AMP liberates Ca^{++} from the sarcoplasmic reticulum, which induces contraction of the myofibrils (Epstein et al., 1971).

Recent investigations have provided new insights into the coupling between the catecholamine-receptor interaction and adenylate cyclase activation. The emerging concept is that these two different macromolecules are in some way integrated in the membrane structure. Sutherland et al. (1962) had observed that the solubilized adenylate cyclase from liver and brain was unresponsive to hormonal stimulation and a similar finding was obtained with the myocardial enzyme (Levey, 1971). Tomasi et al. (1970), in detergent-treated plasma membrane fractions of the liver, demonstrated that

the macromolecule to which NA is bound can be separated chromatographically from the adenylate cyclase activity.

In some cases the hormonal responsiveness of the adenylate cyclase could be restored by the addition of some special phospholipids such as phosphatidylserine or phosphatidylinositol. Lefkowitz and Levey (1972) have observed that a soluble preparation of myocardial adenylate cyclase was activated by NA only in the presence of phosphatidylinositol. This phospholipid was not required for the binding of NA to the receptor site. It was concluded that special membrane phospholipids are involved in the coupling of hormone receptor interaction to the adenylate cyclase activation.

In short, these findings suggest: (a) the β-receptor protein and adenylate cyclase are two different macromolecules; (b) in the functional coupling some membrane phospholipids such as phosphatidylinositol are involved; and (c) the presence of phosphatidylinositol associated with the isolated heart receptor may have a functional importance in the coupling with adenylate cyclase.

1.2.3.2. Muscarinic cholinergic receptor and cyclic 3' - 5' -guanosine monophosphate (Cyclic GMP)

Recent studies seem to indicate that ACh can cause cyclic GMP to accumulate in heart (George et al., 1970; Kuo et al., 1972), brain (Ferrendelli et al., 1970; Kuo et al., 1972) and vas deferens (Schultz et al., 1972). Low concentrations of ACh incubated with heart slices could cause a ten-fold increase in the concentration of cyclic GMP; in cerebral cortex, the increase could reach 3-5-fold (Kuo et al., 1972). More recent studies tend to indicate that this effect is mediated by the muscarinic and not by the nicotinic type of cholinergic receptor. Lee et al. (1972) found that cyclic GMP increased in amount in slices of the cerebral cortex in the rat heart ventricle and in the guinea pig ileum after incubation with such muscarinic agonists as bethanechol, methacholine and pilocarpine; in contrast, tetramethylammonium, a

nicotinic agonist, did not significantly increase the concentration of cyclic GMP. The effect of the agonists was blocked by low concentrations of atropine and other muscarinic antagonists but not by other antagonists of the nicotinic type.

Lee et al. (1972) discuss the significance of these findings and advance the hypothesis that the physiological effects of cholinergic and adrenergic agents on cardiac and intestinal smooth muscle are mediated through the regulation of the relative concentrations of cyclic GMP and cyclic AMP. The cholinergic effects would induce a high cyclic GMP/cyclic AMP ratio and the adrenergic effects would have the opposite result.

The explanation why the nicotinic receptors do not involve the cyclic nucleotides may be that, here, the effect of ACh takes place in a rather explosive way, in milliseconds. On the other hand, the activation of muscarinic receptors is relatively slow and in some cases, it may take seconds to fully develop i.e. the decrease in heart rate, the contraction of the intestinal wall, the secretion by a gland.

1.3. Drug/receptor interactions

1.3.1. Basic steps in drug action

The postulate that drugs produce a response by reacting with specific receptors assumes that a sufficient number of receptors are occupied by drug molecules. To this aim a sufficiently high concentration of the drug has to be present in the direct vicinity of the receptors or of biophase (Furchgott, 1955), also termed receptor compartment (van Rossum, 1966).

Obviously the drug concentration in the receptor-compartment is in some way related to the dose administered to the organism (human or animal) or to the

bath fluid in which an isolated tissue is suspended. In the intact organism the blood or plasma is in contact via diffusion barriers with all parts of the body and therefore, also with the compartment where the receptors are located.

The first step of basic importance in drug action is the relation between the dose administered and the plasma concentration. This relation can be determined by investigations in the field of biomedical sciences, called pharmacokinetics and biopharmaceutics; but since in the strictest sense this relation does not modify drug receptor theories, pharmacokinetics will not be discussed here.

The next basic step is the relation between the plasma concentration and the drug concentration in the receptor compartment for the in vivo situation, or between the concentration in the bath fluid and that in the receptor compartment for the in vitro situation. Since the plasma concentration is a function of time and thus changes after administration of a drug, so the concentration in the receptor compartment changes, but not always in the same direction. The concentration in the receptor compartment has as yet not been determined, so that all studies on drug receptor interactions, as deduced from dose-response or time-response curves, are limited by the lack of information of this relation.

The next step is the drug-receptor interaction proper leading to the generation of a hypothetical stimulus (Stephenson, 1956). Atleast two drug-parameters can be distinguished in this process. These are affinity of the drug for the receptors and the intrinsic activity or the power to generate the stimulus (Ariens, 1954, 1964).

The fourth step presents the production of the stimulus as the ultimate effect. The relationship is extremely complex when a drug acts on receptors in the brain and a change in behaviour is the response. For isolated organs, such as the intestinal smooth muscle, the relation between stimulus and effect may be less

complicated but still so complex that the response is not linearly related to receptor occupation.

The four basic steps of drug action are shown diagrammatically in Fig. 5. The third step is main important step but, however, it is not possible to separate this step from the others, since the analysis of drug receptor interactions relies heavily on dose-response curves and time-response curves.

1.3.2. The affinity of a drug for its specific receptors

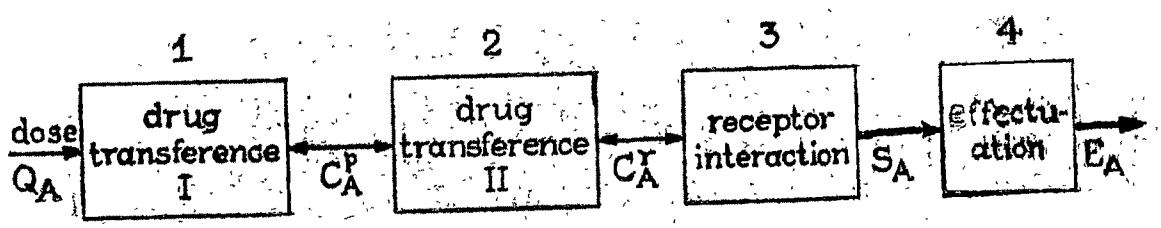
The various substances provoke an effect on a living organism or isolated tissue at extremely low concentrations and this may serve as an argument that the drug is not randomly bound to the tissue or organism but that there are specific receptive substances. Angiotensin is active in concentrations of less than a nanogram in the rat. Natural muscarine is active in a concentration of 10^{-8} molar on isolated smooth muscle, whereas its stereo-isomers are practically inactive.

Certain substances appear to have affinity for receptors in certain tissues whereas slightly altered substances do not fit at all. Drugs reacting with the same type of receptors may differ with regard to their affinity for the receptors. The activity of a drug is, therefore, among other things, characterized by an affinity constant.

The simple version of drug receptor interaction occurs when one drug molecule binds to one receptor according to a reversible process symbolized by the following equation:

$$R + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} RA$$

Fig. 5.



Block scheme representing the basic steps in drug action. The first step gives the relationship between the dose and the plasma concentration (or both fluid concentration). The second step gives the relationship between the plasma concentration and the concentration in the biophase or receptor compartment. The third step is concerned with the receptor interaction, leading to a stimulus which is directly proportional to receptor occupation. The fourth step gives the production of the final effect by the stimulus. This step may be very complicated when psychopharmacological drugs are studied in man. (From Robson and Stacey,1968).

Here R is the symbol for a free receptor, RA the occupied receptor and A a drug molecule receptor occupation is then analogous to a bimolecular reaction, in the binding of a substrate to the active site of an enzyme or in binding of a chemical substance to adsorbing material. Since the receptors are probably a part of membranes or other structures of certain cells of living organ, the analogy with an adsorption process is obvious.

Various binding forces between receptors and drug molecules may be present such as covalent binding forces, electrostatic forces, ion dipoles, hydrogen bonds and hydrophobic binding forces (Pauling, 1960). Drug-receptor interactions are generally reversible processes so that covalent binding is not involved except in some special cases involving non-equilibrium drug action (Nickerson, 1957). The K_A value is experimentally found to range from 10^{-10} moles/l to 10^{-2} moles/l (van Rossum, 1966; Furchgott, 1955, 1966). The K_A value is the quotient of k_2 and k_1 so that a given K_A value may reflect quite different values of the rate constants. Although very few direct estimations have been made of the association and dissociation rate constants, analogies might be drawn with rate constants of chemical reactions and enzymatic processes.

1.3.3. The intrinsic activity

Affinity of a drug for the specific receptors is not alone sufficient to make a drug an agonist. Inspite of having affinity for the same type of receptors drugs may differ in their stimulant action. Acetylcholine is a receptor activator while atropine interacts with the same receptors but acts as a receptor blocker. The extent of the agonistic properties of a drug is reflected by the concept of "intrinsic activity" (Ariens et al., 1957; Ariens, 1964).

Ehrlich (1900) was probably the first to distinguish between affinity and intrinsic action when he stated that drug molecules contain haptophoric groups essential for binding and toxophoric or actophoric groups necessary for toxic or pharmacological action. Clark (1937) too, in his famous monograph, made a clear distinction between these two phenomena. From the observation that certain quanternary nitrogen compounds mimic the action of ACh and thus are synergistic while others cannot do this and so antagonize ACh by blocking specific receptors, Clark concluded that the action of ACh depended on atleast two separate factors; firstly, fixation of the drug by certain receptors and secondly, the power to produce its action after fixation (Clark, 1937).

The difference between agonists and antagonists both acting on the same receptors, might suggest that agonistic activity is an all-or-none property.

The observations that in certain homologous series the agonists differ in agonistic property and the partial agonists have not only agonistic properties, but also behave as antagonists, led Ariens (1954) to the conclusion that there is, in addition to affinity a parameter, intrinsic activity, denoting the power of agonistic action.

Intrinsic activity is thus a parameter giving the degree of activity of a drug when a given number of receptors are occupied. It must be emphasized that intrinsic activity, since it is an abstract concept, applies to any model of drug action, such as the rate theory or occupation theory.

1.3.4. Receptor activators or agonists

On an empty receptor system only drugs that are receptor activators can cause a response. Such drugs are called agonists as e.g. ACh with respect to cholinoceptive receptors and NA with respect to adrenoceptive receptors. Agonists

are characterized by two parameters viz. the affinity constant and the intrinsic activity constant or efficacy constant. Both parameters may vary with alterations in the chemical structure of a parent compound.

Producing a stimulant action does not necessarily imply that the drug in question is an agonist. For instance, atropine causes mydriasis not by activating receptors but, as is generally known, by competing with endogenous ACh. Atropine is obviously not an agonist. Strychnine causes convulsions and, therefore, is classified as a stimulant, but it appears to inhibit the inhibitory synapses and is thus not an agonist. The situation with morphine is less clear. Although there are antagonists available like nalorphine it is not known with certainty that morphine is an agonist. For number of centrally acting substances it is likewise not known whether they are agonists or antagonists.

1.3.5. Receptor blockers or competitive antagonists

Drugs like the higher homologues of ACh, valeryl-and laurylcholine do not activate receptors and consequently do not evoke a response in an isolated piece of intestine. However, they interfere with the action of an agonist and consequently block receptors. The competitive antagonists are merely characterized by the affinity constant.

It has been known for quite some time (Clark, 1937) that competitive antagonists cause a parallel shift of the dose response curves of agonist. The extent of the shift is merely dependent on the dose and the affinity of the antagonist and not on the affinity or the intrinsic activity of the agonist.

Competitive antagonists do not necessarily occupy identical positions on the receptors as the agonists. They generally have large molecules containing hydrophobic moieties, which may bind to sites adjacent to the receptor proper in

such a way that the actophoric group in the molecule does not acquire the correct position for triggering the response (Fig. 6).

The competitive antagonist may occupy a slightly different receptor but there is still competition since simultaneous occupation of the receptors by an agonist molecule and an antagonist molecule is excluded. The situation visualized in Fig. 6 for acetylcholine and an analogue that acts as a competitive antagonist may serve as an explanation for the general observation that affinity goes through a minimum when gradual changes are made in drugs, so that an agonist becomes an antagonist (Ariens, 1964).

1.3.6. Receptor interaction with antagonists

In addition to the agonist-receptor interaction it is important to elucidate the mechanism of action of antagonists, i.e. drugs that will inhibit the response. Gaddum (1937) applied the law of mass action to the case in which two drugs reversibly compete for a receptor site. He found that, in some cases, increasing concentrations of the antagonist produced a displacement towards the right of the dose-response curve of the agonist, without changing the slope or the maximal response; this defines the antagonism as competitive (Fig. 7a). This should be differentiated from the noncompetitive type of inhibition in which there is a non-parallel displacement with depression of the maximal response (7b).

If two drugs A and B compete for a single population of receptors, the equation describing the occupancy of the agonist will be

$$-{}^{*}_{Y_A} = \frac{(\overset{*}{A})/K_A}{(\overset{*}{A})/K_A + (\overset{*}{B})/K_B + 1} \qquad (1\text{-}1)$$

Fig. 6

Model of the interaction of an agonist (acetylcholine)with a receptor and of an antagonist (cyclohexyl phenylglycolic ester of choline) with adjacent parts of the receptor leading to a wrong presentation of the onium head. The correct presentation of the onium head is supposed to be essential for agonistic action (From Robson and Stacey, 1968).

FIG. 6

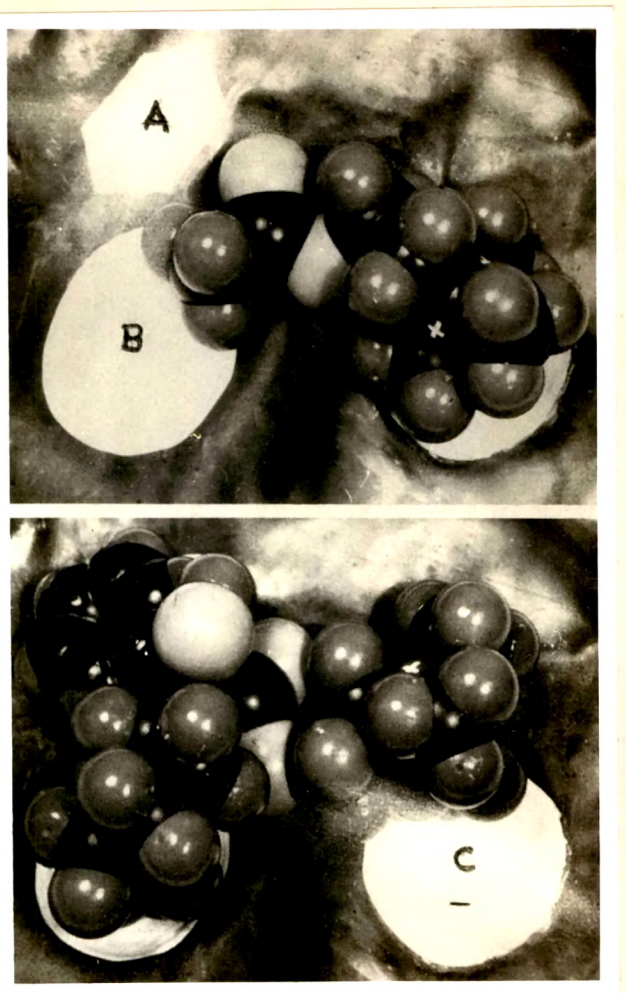
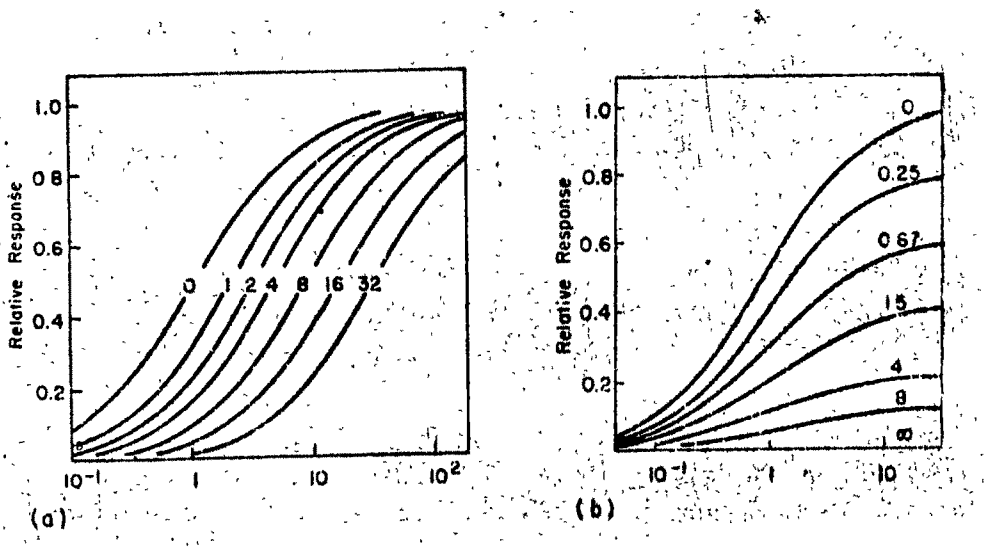


Fig. 7.



(a) Theoretical concentration-response curves of an agonist in the presence of various concentrations of a competitive antagonist. (b) The same as (a) but here the inhibitor is noncompetitive. (From Ariens, 1964).

Here the asterisk denotes the simultaneous presence of A and B. Suppose that, at a fixed effect, the agonist (A) alone produces the same occupany as the agonist in the presence of the antagonist ($\overset{*}{A}$); this equates both sides of equation (1 - 1). Then the dose ratio $\overset{*}{A}/A = X$, where

$$X = \frac{\overset{*}{(A)}}{(A)} = \frac{\overset{*}{(B)}}{K_B} \doteq 1 \qquad (1\text{-}2)$$

$$X - 1 = \frac{\overset{*}{(B)}}{K_B} \qquad (1\text{-}3)$$

This shows that if the effect produced by the agonist is a function of $\bar{Y}_A$, the introduction of the antagonist will simply shift the log-concentration curve to the right without changing the amplitude of the slope. If log (x-1) is plotted against log $(\overset{*}{B})$, then a straight line is obtained in which the intercept with the abscissa corresponds to K_B, i.e. the equilibrium constant of the antagonist.

When the Hill number is higher than 1, then for the occupancy of the antagonist we will have

$$\bar{Y}_B = \frac{\overset{*}{(B)}^m}{\overset{*}{(B)}^m + K_B} \qquad (1\text{-}4)$$

and Eq. (1-3) will take the following form

$$x^n - 1 = \frac{\overset{*}{(B)}^m}{K_B} \qquad (1\text{-}5)$$

In this case the logarithmic plot will be nonlinear, having a slope of m at low dose ratios and of m/n at high dose ratios.

Schild (1957) introduced the concept of pA_x, to quantitatively define competitive antagonism

$$pA_x = -\log \overset{*}{(B)} \qquad (1\text{-}6)$$

pA_x is defined as the negative logarithm of the molar concentration of the antagonist, which gives a dose ratio of x.

From Eq. (1-3) it can be deduced that

$$pA_x = \log K_B - \log (x-1) \qquad (1\text{-}7)$$

and, when $x = 2$

$$pA_x = pA_2 = \log K_B \qquad (1\text{-}8)$$

The determination of pA_2 is not only of theoretical but of practical importance, since it is a good way of determining if a drug is a reversible competitive antagonist. Furthermore, it permits a more accurate calculation of K_B, and it allows us to compare receptors in different tissues.

1.3.7. Characterization of drug effects

The effects of drugs are variously expressed as biochemical or physiological changes, or as the occurrence or relief of clinical symptoms. Thus, drugs are described as anticoagulants, vasoconstrictors, psychotomimetics, analgesics and so forth. However, for evaluation and comparison of drugs, their effects must be related to dosage and they must be further characterized in terms of maximum efficacy, variability and selectivity. Although interdependent, intensity of drug effect and time course of drug effect (latency, peak time and duration) should be considered separately.

1.3.7.1. The dose-effect relationship

Intensity of drug effect usually refers to the peak effect after a single dose or to the average effect during chronic administration. There is no one, characteristic relationship between intensity of drug effect and drug dosage. A dose-effect curve may be linear, concave upward, concave downward or sigmoid. Moreover, if the observed effect is the composite of several effects of the drug,

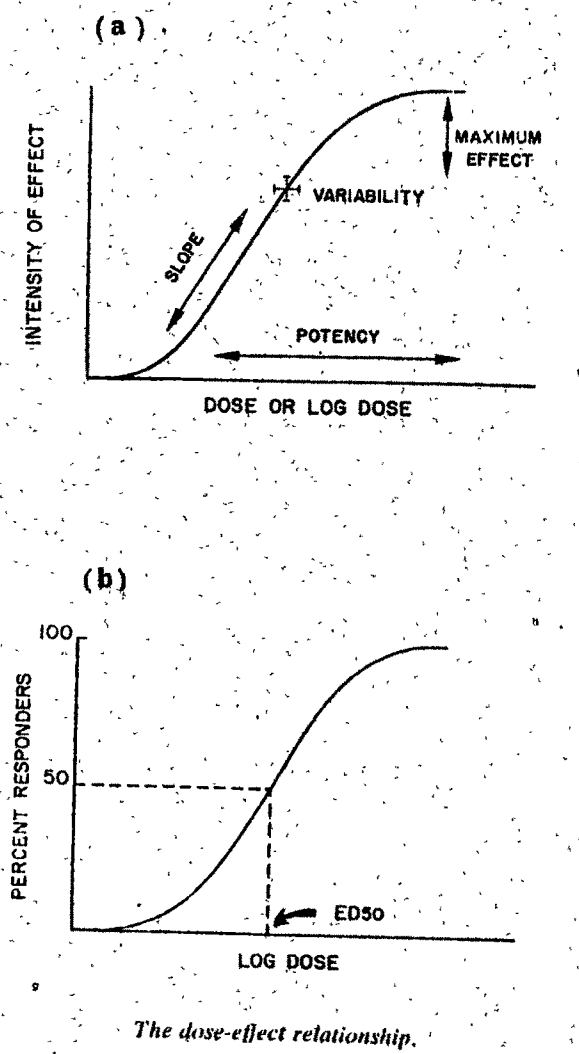
such as the change in blood pressure produced by a combination of cardiac, vascular and reflex effects, the dose-effect curve need not be mono-tonic. However, a composite dose-effect curve can usually be resolved into simple curves for each of its components; and simple dose-effect curves, whatever their precise shape, can be viewed as having four characterizing parameters; potency, slppe, maximum efficacy and variability. These are illustrated in Figure 8 for the common sigmoid log dose-effect curve. The logarithmic transformation of dosage is often employed for the dose-effect relationship, because it permits display of a wide range of doses on a single graph and because it has certain mathematical advantages when dose-effect curves are compared.

The location of dose-effect curve along the dose axis is an expression of the potency of a drug. Potency is influenced by the absorption, distribution, biotransformation and excretion of a drug as well as being determined by its inherent ability to combine with its receptors. For therapeutic applications, the potency of a drug is necessarily stated in absolute dosage units; for comparison of drugs, relative potency is more convenient expression.

The slope of the more-or-less linear, central portion of the dose-effect curve is more of theoretical than practical importance; however, it can have theraputic significance. For example, the steep dose-effect curve for CNS depressant implies that there is a small ratio between dose that produces mild sedation and that which causes coma and that excessive or inadequate effect may occur if the dose of the drug is not carefully adjusted.

The maximum effect produced by a drug, even at very large dosage, is termed its "ceiling effect" and is referred to as its maximum efficacy. Maximum efficacy of a drug may be determined by its inherent properties and be reflected as a plateau in the dose-effect curve, but it may also be imposed by other factors.

Fig. 8.



The dose-effect relationship.

(a) Representative dose-effect curve, illustrating its four characterizing properties (see text for explanation). (b) Representative dose-percent curve illustrating the median effective dose (ED_{50}). (From Fingl and Woodbury, 1965).

Maximum efficacy of a drug is clearly one of its more important characteristics. Efficacy and a potency of a drug are not necessarily correlated and these two characteristics of a drug should not be confused.

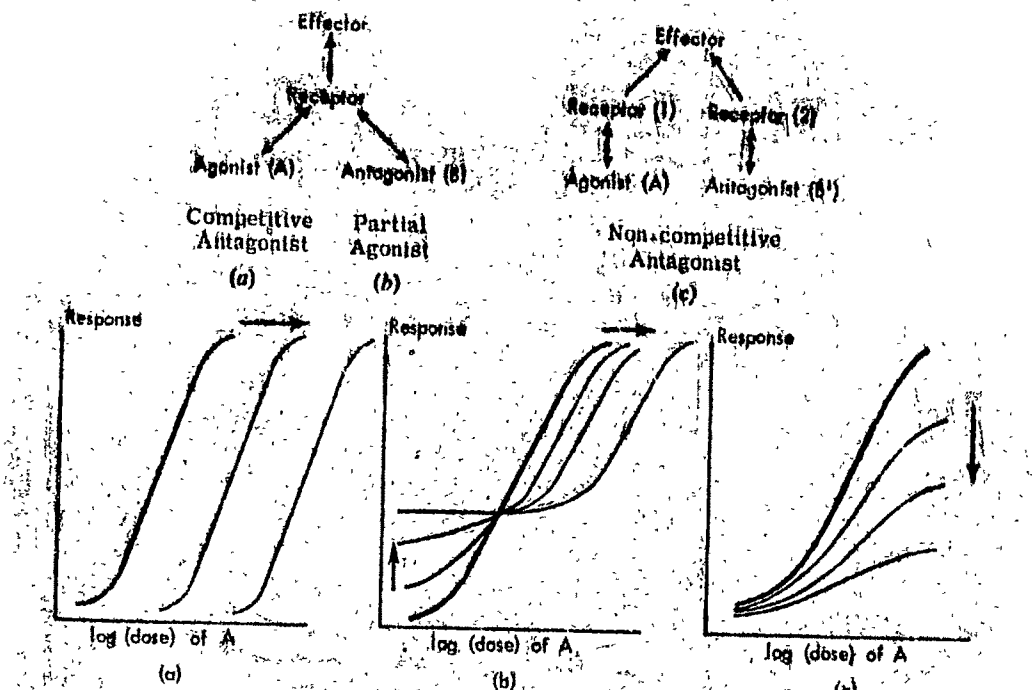
When all known sources of variation are controlled or taken into account, drug effects are never identical in all patients or even in a given patient on different occasions. The perpendicular brackets in Figure 8 indicate that biological variation of the dose-effect relationship can be visualized in either of two ways. The vertical bracket expresses the fact that a range of effects will be produced if a given dose of a drug is administered to a group of individuals; alternatively, the horizontal bracket expresses the fact that a range of doses is required to produce a specified intensity of effect in all individuals.

1.3.7.2. Drug antagonists

A competitive antagonist has an intrinsic activity of zero; it shifts the dose-response curve to the right and the inhibition it causes can be removed by increasing the concentration of agonist (Fig. 9a).

A compound with a high affinity and a low or moderate intrinsic activity will also be capable of reducing the effect of an agonist. The inhibition will again be removed by increasing the concentration of the agonist but the antagonist will itself be capable of producing a response in the preparation. Such a compound is consequently described as a partial agonist. It is also sometimes known as a dualist because it behaves as both an agonist and an antagonist. The effect of a partial agonist on the dose-response curves of an agonist is illustrated in Figure 9b. The form of the curves is simply explained, atleast qualitatively. In the presence of very small doses of the agonist, the response obtained is determined almost entirely by the partial agonist. This response

Fig. 9.



In all the diagrams the dose-response curve of the agonist in the absence of antagonist is represented by the bolder line and the arrow indicates the direction of increasing concentration of the antagonist.

Dose-response curves of an agonist in the presence of different types of antagonists. (From Lewis, 1970).

will increase as the concentration of the partial agonist is increased until it is exerting its maximum effect which is determined, of course, by its intrinsic activity. As the concentration of agonist is increased in the presence of a constant concentration of the partial agonist, its effects becomes progressively more dominant and the dose-response curve takes on the form characteristic of that of the agonist although, because of the continuing presence of the partial agonist, the curve is displaced to the right. It will be seen that in the presence of high doses of the partial agonist the response of the preparation remains constant over a range of concentrations of the agonist. This is a reflection of the fact that under these conditions the agonist is ineffective until it is present in a concentration sufficiently high to enable it to free some of the receptors by displacement of the antagonist. It can also be shown that there is a dose of agonist which will always produce the same response, irrespective of the concentration of partial agonist. This explains why the dose-response curves in Figure 9b have a common point of intersection. It should be noted that some substances are capable of combining irreversibly with drug receptors. They reduce the number of receptors available to the agonist and they cannot be displaced by high concentrations of the latter.

A non-competitive antagonist is one which operates on a different population of receptors from those activated by the agonist but which can nevertheless alter the response of the effector system. A non-competitive antagonist depresses the response to the agonist to an extent dependent on the concentration of antagonist. Because the agonist and antagonist affect different receptor systems, the inhibitory effect of the antagonist is independent of the concentration of agonist and no amount of the latter, however, great, will overcome the inhibition. To put it in another way, the agonist is incapable of evoking the maximum response when no antagonist is present-in effect, its intrinsic activity has been reduced.

Dose-response curves in the presence of a non-competitive antagonist are illustrated in Figure 9c. Some non-competitive drugs increase the response of a tissue to the agonist. They are sensitizers rather than inhibitors.

1.3.8. The pA_x, pD_x and pD'_x scales

Competitive antagonists can be quantitively compared on the basis of their pA_x values, an index of activity devised by Schild (1947). It is usual to measure the pA_2 value which is the negative logarithm of the molar concentration of the antagonist which results in the dose of agonist having to be doubled in order to produce the effect it had in the absence of the antagonist. The pA_{10} and, more rarely, other pA_x values may have to calculated and pA_x can be generally and succintly defined as the negative logarithm of the molar concentration of the antagonist required to reduce the effect of a multiple dose (x) of the agonist to that of a single dose. The more active an antagonist is the smaller will be the dose required to reduce the effect of the agonist and the larger, therefore, its pA_x values. The pA_x scale was introduced on purely empirical grounds as a convenient index of antagonistic activity but it fits very well into the framework of modern receptor theory.

Competitive antagonists, as has been seen, in effect reduce the affinity of an agonist for its receptor and it can be shown that the pA_2 value is proportional to the affinity of the antagonist for the common receptors.

1.3.9. Measurement of pA_x values can be put to practical use in several ways

(i) Dose-response curves obtained in the presence of different concentrations of a competitive antagonist are parallel with one another and with the curve obtained in the absence of the antagonist. pA_x values are, therefore, independent

of the concentration of agonist used in their calculation. If, in a particular instance, a pA_x value is found to increase with the dose of agonist, it must be concluded that the antagonism is non-competitive in type.

(ii) The Ariens theory predicts that $pA_2 - pA_{10}$ will be 0.95 for any competitive antagonist. This relationship can be used to establish whether a particular agent is acting as a competitive antagonist. For a number of practical and theoretical reasons, a consistently close agreement with the predicted figure cannot be expected but if $pA_2 - pA_{10}$ is of the order of 0.5 or less, it can be concluded that competitive antagonism is not being measured. Some indubitably competitive antagonists exert, in addition, a measure of non-competitive block at high concentrations. For this reason, the amount of drug which is required to bring about a tenfold reduction of the effect of the agonist is rather smaller than would be expected. Consequently, pA_{10} is larger and $pA_2 - pA_{10}$ is smaller that required by theory. The intervention of non-competitive antagonism into the action of a competitive antagonist can be suspected if its $pA_2 - pA_{10}$ values though greater than 0.5, is appreciably less than 0.95. In this event, tests for non-competitive antagonism should be applied with the antagonist present in a high concentration.

(iii) An agonist may act on a number of different tissues, on each of which it may be inhibited by the same competitive antagonist. The pA_x value for the antagonist should be the same for all tissues bearing the same type of receptor. An actual example will illustrate this. The pA_{10} values for atropine against acetylcholine on a number of tissues were found to be 8.1 with guinea-pig ileum, 7.6 with guinea-pig lung, 8.1 with rat intestine, 8.3 with frog atrium and 4.2 with frog rectus (Arunlakshana & Schild, 1959). These results provide a clear indication that the receptors in the frog rectus muscle differ from those in the

other tissues and that the latter are identical one with another. In this particular instance, it was, of course, already known that this was so (the receptors in the rectus muscle are nicotinic in type while the other tissues carry muscarinic receptors: but measurement of pA_x values might well throw light on the nature of receptors whose identity has been less certainly delineated than those of the acetylcholine system.

(iv) A number of substances may all evoke a response in a particular preparation. In order to determine which of these are operating through a common receptor system, a competitive antagonist active against all the agonists is sought and a pA_x value against them all is calculated. This should be the same for each agonist - antagonist combination which affects the same receptor type.

The activities of non-competitive antagonists are measured on the pD'_x scale. The pD'_x value is the negative logarithm of the molar concentration of the antagonist which is required to reduce the maximum effect of the agonist to 1/x of that which it exerts in the absence of the antagonist. The pD'_x value is analogous to the pD_x value which is applied to agonists and which is defined as the negative logarithm of the molar concentration of the agonist which produces an effect equal to 1/x that of the maximum response. As in the case of the pA_x scale, theoretical and practical considerations indicate that the pD'_2 and pD_2 values are the measures of choice. It will be recalled that the concentration of agonist required to produce half its maximum effect is inversely related to the drug affinity. From this relation it will be clear that the pD_2 value is directly proportional to the logarithm of the affinity of an agonist and the pD'_2 value is similarly related to the affinity of the non-competitive antagonist for its particular receptors. It is a logical measure of antagonistic activity

because the more avidly the drug seeks its own receptors the greater will be its action on the common effector system and the greater the hindrance offered to the action of an agonist on that system.

Like the pA_x value, the pD'_x value should be independent of the dose of agonist used in its measurement, provided that the antagonist is acting in a truely non-competitive way. If it is found in an individual case that pD'_x falls as the dose of agonist increases, it must be concluded that the antagonist is behaving wholly or partly in a competitive way, the higher dose of agonist partially reversing the inhibition imposed by the antagonist. The effect of agonist concentration on pA_x and pD'_x values is summarized in Table 2.

The pD'_x values of non-competitive antagonists can be put to the same kind of practical use as are pA_x values. Thus papaverine is a non-competitive antagonist and atropine a competitive antagonist of a number of agents which cause contraction of the guinea-pig ileum. The pA_2 and pD'_2 values of atropine and papaverine respectively against three agonists (barium chloride, histamine and an acetylcholine analogue) are displayed in Table 3 and Figure 10. The pD'_2 values for papaverine are approximately the same against all three agents but the pA_2 values differ. It can be concluded that the receptors for barium chloride, histamine and acetylcholine are dissimilar but that they all operate through a final common mechanism which is inhibited by papaverine. Results such as these, incidentally, illustrate how inaccurate it is to speak of specific antagonists: atropine has some action, albeit a small one, on the histamine and barium receptors as well as those which respond to acetylcholine.

1.3.10. Isolation of the receptors

The techniques involved in the isolation of receptors are somewhat

Table 2. The effect of increasing the dose of agonist on pA_x and pD'_x values.*

	pA_x	pD'_x
Competitive Antagonist	No effect	Decreases
Non-competitive Antagonist	increases	No effect

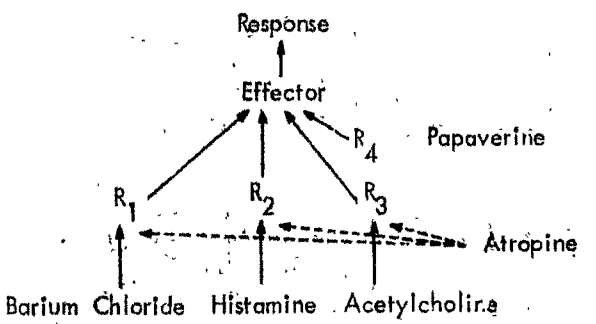
* (from Lewis, 1970).

Table 3. The effect of antagonists on the response of the isolated guinea pig ileum.*

	Agonist		
	Barium Chloride	Histamine	Acetylcholine Analogue
pA_2 of atropine	3.2	6.5	8.1
pD_2^1 of papaverine	5.0	4.9	5.3

* (from Ariens, 1964).

Fig. 10.



Analysis of the sites of action of agonists and antagonists deduced from the data of Table 2. (From Lewis, 1970).

similar to those used for the enzymes, a field of biochemistry in which so many extraordinary advances have been made. There are important differences, however, that make the isolation of receptors considerably more difficult. In general, the activity of an enzyme can be assayed in vitro at the different stages of isolation and purification, since the enzyme remains essentially the same. In the case of the receptors, as soon as the tissue is homogenized for cell fractionation, the integrity of the cell is lost and with this, the pharmacological criterion of response is no longer available. In the study of receptors, binding with labelled drugs is the most common procedure. Binding can be followed in various subcellular fractions, particularly in membrane fragments and an affinity constant can be obtained and compared with the constant found with traditional pharmacological procedures on intact tissue (Arunlakshana and Schild, 1959; Furchgott, 1967). Even if an affinity constant is obtained in the in vitro interaction of the ligand with a membrane or with an isolated protein molecule, we should not expect it to coincide with the affinity constant for the intact tissue.

The following factors may change the affinity constant of a receptor in vivo or in vitro: (a) In vivo, binding of the ligand to the receptor macromolecule may be modified by the process of diffusion to and from the cell membrane. This modification has been thoroughly discussed by Furchgott (1955, 1964) in his concept of the "biophase". On the other hand, in vivo, there may be metabolizing enzymes and sites of nonspecific binding that may not be present in vitro (i.e. in an isolated receptor macromolecule). This complicates comparison with the isolated receptors. (b) another important difference may result from the fact that, after isolation, the receptor macromolecule may have a different conformation than when it was integrated in the membrane structure. This may also affect the affinity constant for the ligand. (c) some neurotransmitters may release other biologically active substances, which may affect

the biological response. (d) According to the concepts of Stephenson (1956), the pharmacological potency of an agonist does not necessarily reflect its chemical affinity for the receptors; it may mainly reflect its capacity to induce a response, the so-called stimulus.

Essentially, two basic techniques are used to monitor the isolation of receptor macromolecules. In one, reversible ligands are used on subcellular and protein fractions obtained from the tissue containing the receptor and affinity constants are measured; competition experiments are also carried out to determine the degree of the specificity of the binding. This type of investigation has all the shortcomings mentioned earlier. In the other, a covalent type of binding is introduced so as to permanently label the receptor before isolating it from the tissue.

1.3.11. The theories of drug action

1.3.11.1. The occupation theory of drug action

Agonists are receptor activators, whereas competitive antagonists only occupy receptors without producing activation and consequently are receptor blocking agents.

It has been stated that the pharmacological response is in general a function of the number of receptors occupied. In the so-called occupation theory it is assumed that receptor activation by an agonist occurs as long as the receptor is occupied by an agonist molecule.

Since the rate constant for association (k_1) is probably very large for most drugs, it is unlikely that the development of response as a function of time can be due to rate of receptor occupation. It is more likely that the rate of

penetration of the drug into the biophase or receptor compartment is rate limiting (Furchgott, 1964). If the drug/receptor occupation and dissociation process is fast with respect to diffusion in and out of the biophase, there is always equilibrium at the receptor level and kinetic studies cannot then provide information on the validity of the occupation theory.

The situation may be different for very potent drugs (very low K_A value). Since theoretically k_1 values larger than 10^9 l/mole sec. are not possible, small k_2 values will necessarily be found. In some tissues, therefore, the dissociation rate constant k_2 might be smaller than the diffusion rate constants and, therefore, receptor occupation might be rate limiting. Paton and Rang (1965) have presented evidence that the k_2 of atropine is around 10^{-3}/sec. This would imply that atropine would act in an irreversible manner.

1.3.11.2. The rate theory of drug action

The response of a drug as function of time is in some instances a rapid contraction followed by some relaxation. This effect is most obvious with nicotine acting on autonomic ganglia (Paton and Perry, 1953), in the guinea pig intestine and also with depolarizing curariform drugs such as decamethonium and succinylcholine acting on the motor-endplate (Katz and Thesleff, 1957). The fading effect that can be observed in time-response curves is difficult to interpret using the occupation theory, assuming that the fade phenomenon is indeed due to drug-receptor interaction.

Following a good deal of original thinking and experimental investigations, a new theory of drug receptor interaction has been formulated by Paton (1961). The basic idea is that the response of a drug is not a function

of the number of receptors occupied, but of the rate of drug receptor association (Rang, 1966).

The rate of association of drug A with the receptors depends on the number of free receptors, the drug concentration and the association rate constant K_1 (Paton, 1961).

The rate theory has stimulated much thought and revived the interest in basic problems of drug/receptor interactions (Furchgott, 1964; Paton and Rang, 1966; Belleau, 1965). There are, however, no experiments yet from which it can be concluded that drug response is governed by the rate of drug/receptor association. At the same time there is no definite experimental evidence that the rate theory is not valid. The high degree of stereospecificity (e.g. for muscarine) in contrast to symmetrical drugs such as tetramethylammonium represents an argument against the rate theory (Belleau, 1965).

Occupation theory and rate theory are probably not exclusive alternatives but the rate theory may be valid for certain types of receptors and the occupation theory for others (Furchgott, 1964).

1.3.11.3. The dissociation-rate theory

It is implicit in the rate theory of Paton that an agonist can achieve a fast rate of association with the receptor only if the concentration of the agonist molecules and the dissociation rate constant are high. Paton and Rang (1966) suggested the possibility that it might be that drug dissociates quickly just because it is an agonist. This alternative receptor theory is consistent with data of Gill (1965).

1.3.11.4. The flux-carrier hypothesis

Mainly because curariform drugs such as decamethonium and suxamethonium

first cause depolarization and initial contraction and thereafter muscle paralysis Mackay (1963) was led to postulate the flux carrier hypothesis. Mackay assumes that the drug is transported through the cell membrane by a carrier mechanism. The influx of drug is initially large when the carrier is not yet saturated and then decreases gradually to a steady state condition.

This theory is closely analogous to the rate theory and may serve as a concrete although restricted model of the drug receptor interaction.

1.3.11.5. The conformational perturbation theory

The drug parameters, intrinsic activity and affinity do not in essence provide information about the physico-chemical entities responsible for the mechanism of drug action. This lack of physical basis was one of the reasons why Belleau put forward the conformational perturbation theory of drug action (Belleau, 1964, 1965).

Belleau (1964, 1965) has provided a large body of information on physical entities allowing a thermodynamic analysis of drug receptor interaction. He has provided good evidence that nonpolar groups, alkyl chains etc. in the drug molecule may bind to non-polar parts of the receptor surface. An important conclusion reached by Belleau is that number of bound water molecule bind with a receptor.

1.3.11.6. The dynamic receptor hypothesis

In their study of the mechanism of action of α-and β-adrenergic substances Bloom and Goldman (1966) postulated the dynamic receptor hypothesis. They consider receptors as active sites of an enzyme (such as Mg^{++} - ATPase) occupied to a certain extent by endogenous substrate ATP. An agonist like Adr may

react with this enzyme-substrate complex and so facilitate the rate of enzymic hydrolysis of the substrate. Such a situation differs clearly from the occupation theory and rate theory. Equilibrium cannot be reached, but at the best only a steady state condition.

If there is excess substrate the situation is analogous to the equilibrium situation in the case of the occupation or rate theory (agonism, partial agonism and competitive antagonism).

It is obvious that when the substrate concentration is rate-limiting, desensitisation occurs after the action of an agonist. The substrate concentration decreases so that the number of 'receptors' for the agonist decreases also.

It can be demonstrated that decrease of substrate is analogous to decrease of receptors by dibenamine or other irreversibly acting drugs. Spare receptors may, in view of the dynamic receptor hypothesis, also signify spare substrate.

1.3.12. Structure-activity relations

From structure-activity studies a number of important practical conclusion can be drawn irrespective of the validity of any drug-receptor theory. For instance, correlation between substituents and biological activity as made by Hansch et al. (1965) may lead to predictions of the structure of new drugs. However, if the aim is to study which moieties in the molecule are essential for a specific biological action and affinity, it is necessary to know with what category of receptors the drug molecules interact and to know the value of the dissociation constant. A thermodynamic analysis of drug action can only be meaningful if the biological activity is expressed in parameters such as the dissociation constant and intrinsic activity constant instead of ED_{50} values of equipotent doses (Ariens, 1964).

1.4. Species difference and pharmacological actions of drugs

1.4.1. General considerations

There is a considerable species variation in regard to the pharmacological actions of drugs. Intracerebro-venticular (ICV) injection of a drug which caused hyperthermia in one species, may cause hypothermia in another and, have no action in the third. For example, ICV injection of NA produces decrease in temperature by 1-2°C in cats (Feldberg and Myers, 1965) and increase in temperature by 1-3°C in rabbits (Cooper et al., 1965; Fuchebusch et al., 1965). In rats there is increase in temperature with small dose (<6 ug) of NA and decrease in temperature with higher dose (>10 ug) of NA (Feldberg and Lotti, 1967). On the other hand, dose-dependent increase in temperature was obtained by Myers and Yaksh (1968) in rats. ICV injection of 5-HT produced decrease in temperature by 0.4°C in rabbits (Cooper et al., 1965; Jacob and Peindaries, 1973); in rats there was dose-dependent increase (Crawshaw, 1972) or decrease in temperature (Feldberg and Lotti, 1967; Myers and Yaksh, 1968) while in cats there was a variable response (Feldberg and Myers, 1964; Kulkarni, 1967). ICV injection of carbachol induces an increase in body temperature in cat (Hall, 1972) and a decrease in rabbit (Bligh and Cottle, 1971) and in rat (Meeter, 1971). Others found dose-dependent increase in temperature in rat (Crawshaw, 1972).

The reactivity of the guinea pig to the contraceptive combined treatment is quite different from that of mouse and rat. The guinea pig is very sensitive to the antifertility action of the contraceptive drugs but it is unresponsive to their microsomal enzyme-inducing activity (Briatico et al., 1976).

The actions of prostaglandins (PG) are also found to be variable in different species. PGE_1 potentiates Adr-induced contractions of the vas deferens of rabbit (Horton et al., 1963; Manteganza and Naimzada, 1965) and guinea pig

(Mantegazaa and Naimazada, 1965) but inhibits these contractions in rat (Clegg, 1966a). PGE_1 inhibits the inhibitory action of Adr on carbachol-induced spasm of guinea pig tracheal chain preparation (Clegg, 1966b). $PGF_{2\alpha}$ raises the blood pressure in dog, rat and spinal chick (Horton and Main, 1967; Du Charne and Weeks, 1967), but lowers it in cat and rabbit (Anggard and Bergsterom, 1963). PGE_1 in doses of 0.01-1.0 µg/ml of perfusion fluid has a marked and usually long-lasting inotropic and chronotropic effect on isolated guinea pig heart, but has no effect on rabbit, cat or rat heart prepared according to Langendorff or (Berti et al., 1965; Vergroesen et al., 1967). In high doses PGE_1 has a negative inotropic effect on the rabbit heart (75 ug) and a positive inotropic effect on rat heart (10 ug; Berti et al., 1965).

Differing Variable responses to drugs in various ~~the different~~ species may be due to different metabolism of the drug. For example, pethidine is rapidly transported in the dog and hence has little depressing action in this species. Phenylbutazone is rapidly metabolized in guinea pig, rabbit and dog and hence enormous doses are required to induce anti-inflammatory effect for which it is utilized in man (Grollman, 1965). Morphine depresses man, dog and rat but it stimulates cat and probably mouse. The subcutaneous fatal dose of morphine per unit body weight in mouse is 1½ times that of dog, 2 to 3 times that of rabbit, 8 to 15 times that of cat and about 100 times that of man (Ghosh, 1971). Following the same dose of hexobarbitone per unit body weight, the average sleeping time of rat is about 7 times that of mouse and in dog its effect lasts for hours. Metabolic rate of ethanol was found to be different in different species: (in mg per kg of body weight per hour) 90-180 in man, 100-230 in dog, 150-180 in cat, 100-200 in rabbit, 170-540 in rat and 370-1500 in mouse (Jorge, 1963).

Mammals

Guinea pig and man are 500 times more sensitive to histamine than rat and mouse. Histamine powerfully contracts the uterus of guinea pig while it relaxes that of rat. The rat heart is known to be very resistant to cardiac glycosides. Adr produces inhibition of rat uterus whether pregnant or not but it produces relaxation of the non-pregnant cat uterus and a contraction of the cat uterus in early stages of pregnancy and also of the rabbit uterus while in pregnant and non-pregnant dog, both stimulation and inhibition may occur. Alloxan produces diabetes in a number of species but not in guinea pig. Dog cannot acetylate sulphonamides effectively. Following prolonged administration of pronethalol, lymphoid tumours (thymemas) have been produced in mouse but not in rat or guinea pig. Penicillin is 100 to 1000 times more toxic to guinea pig than mouse. Rabbit generally shows shock ~~symptoms~~ signs following insulin when blood sugar level is 45 mg per cent, while dog shows ~~symptoms~~ signs only when the level is about 18 mg per cent (Ghosh, 1971).

1.4.2. Drug metabolism and species difference

The knowledge of species difference in drug metabolism is very useful to predict the pharmacological and toxicological properties of a given compound in man from experimental data obtained in animals. Such studies also have yielded valuable new biochemical information of quite fundamental nature. Examples of this type concern acetyl CoA, the role of pyridine nucleotides in oxygen incorporation, human genetics, phylogenic and ontogenetic enzyme development, intracellular structure and membrane function, and biosynthesis of vitamin C (Brodie, 1962). Rat, rabbit and mouse metabolize imipramine by N-demethylation but the rate of metabolism of desmethylimipramine is much slower in rat, a species in which imipramine is more active. Species differences in the metabolism of benzyl-N-benzyl carbethoxyhydroxamine, an agent affecting lipid metabolism, have also been observed (Edelson et al., 1968). An unusual metabolite,

the O-benzylbenzaldoxime, is formed by man and the rat but not by monkey, cat or guinea pig. Considerable species difference in mammalian nitro reduction is reported (Fouts and Brodie, 1957), the enzyme being highly active in mouse, guinea pig and rabbit and less so in rat and dog. The lack of acetylation capabilities in the dog of the long-acting sulphonamide, sulphsomizole, is observed which is acetylated in man, rat and rabbit (Bridges and Williams, 1963). Similarly, species difference for the metabolism of several groups of drugs such as organic mercurials, indoles, alkaloids, esters, thio-compounds etc. are observed by various groups of workers. Some of the factors like exterral environment, difference in binding of drugs either to tissues or to plasma compounds are essential to corsider for comparing the metabolism of drugs in different species (Borga et al., 1968 ; Sturman and Smith, 1967). More obvious are two other factors, the concentration and type of drug-metabolizing enzymes in each species. Marked species variations have been shown in both K_m and V_{max} for the metabolism of ethylmorphine (Castro and Gillette, 1967) in liver microsomes of rat, mice, guinea pig, rabbit and monkey (African green). Williams (1967) stated that aromatic hydroxylation varies quantitatively and qualitatively in haphazard fashion among species and it is not wise to get conclusion of drug toxicity from metabolism in animals and utilization of it to human being. Brodie and Reid (1967) also support this view. Therefore, it is wise to study drug metabolism in higher non-human primates such as chimpanzee, orgar-utan and gorilla.

Or, better still, in man!!