THE EFFECTS OF CERTAIN CENTRAL NERVOUS SYSTEM STIMULANT DRUGS AND RELATED COMPOUNDS ON THE *IN VITRO* OXYGEN UPTAKE AND ENZYMIC ACTIVITY IN THE RAT BRAIN.

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Conventions for Citing References

The conventions used in this thesis are those of the Journal of Physiology. Where journal abbreviations did not appear in the "Suggestions to Authors" (J. Physiol. (1960) 150, 1-33), resort was made to "World Medical Periodicals", (published by the World Medical Association, New York, 1957).

INTRODUCTION

GENERAL INTRODUCTION

Since the dawn of history, man has sought to modify his level of consciousness and alter his mood by means of drugs. A variety of substances, including opium, coca, hemp, peyote and alcoholic beverages have been, and still are, used throughout the world to induce hallucinations and states of euphoria (Goodman & Gilman, 1955). However, despite man's long established interest in substances affecting the mind, his attempts to treat abnormal mental conditions by means of drugs, have, until recently, been haphazard and largely unsuccessful (Marley, 1959). The discovery, within the last twenty years, of drugs having specific effects on the higher functions of the central nervous system, has therefore been an event of major importance to psychiatry. Although the improvement afforded by psychotropic drugs is probably entirely symptomatic, their value in the treatment of a number of abnormal mental conditions has now been established.

While numerous attempts have been made to classify psychotropic drugs, none of the proposed systems is completely satisfactory (Jacobsen, 1959;

Shepherd & Wing, 1962). For the purpose of this thesis, which is concerned primarily with drugs used in the treatment of depressive illnesses, the clinically-useful compounds will be divided into two main categories, namely, the tranquillisers and the antidepressives. Whereas the tranquillisers are employed in the treatment of conditions characterised by symptoms of anxiety, violence and overactivity, the antidepressives are used in the treatment of mental apathy and depression (Lewis, 1962). An additional group, the psychotomimetics, is of limited clinical importance and although some of the drugs in this category have been used in psychotherapy (Osmond, 1957), it seems likely that their main value will be in the investigation, rather than in the treatment of mental illness (French, 1958).

Although a large number of antidepressives are in widespread use today, none is entirely free from undesirable side-effects and indeed some are of very limited therapeutic value. Thus, the amphetamines, as well as causing habituation, anorexia and side-effects arising from their sympathomimetic activity, may precipitate hysterical episodes (Connell, 1958). Although dimethylamino ethanol has been claimed to be

of value in the treatment of mild depression, the evidence for this is inconclusive (Brodie, Sulser & Costa, 1961; Rees, 1960), while iproniazid may produce postural hypotension (Sjoerdsma, Gillespie & Udenfriend, 1959) and liver necrosis (Katz, Klinger, Silva, Rodriguez & Ducci, 1959; Pare & Sandler, 1959; Fisher & Oliver, 1958). Imipramine, as well as having atropine-like side-effects, may cause death due to circulatory collapse following its use after the prolonged administration of a monoamine oxidase blocking drug (Davies, 1960).

Until very recently, the availability of antidepressive drugs has been dependent upon the clinical observation of the central stimulatory side-effects of drugs intended for the treatment of other conditions. Thus, although amphetamine sulphate was synthesised as a sympathomimetic in 1927 (Alles, 1927), it was not until 1936 that it was used clinically in the treatment of mental depression (Peoples & Guttman, 1936). A similar pattern of development has followed the introduction of other compounds, such as the anti-tubercular drug, iproniazid (Fox & Gibas, 1953;) and also imipramine which was synthesised as a potential tranquilliser (Kuhn, 1957).

In an attempt to rationalise the synthesis and evaluation of antidepressive drugs, the correlation of monoamine oxidase inhibition with central stimulant activity has been valuable (Zeller, Barsky & Berman, 1955; Zeller, Barsky, Fouts, Kirchheimer & Van Orden, 1952). While this approach has led to the synthesis of a number of useful compounds, including phenelzine and tranylcypromine, it is limited in its application, since not all of the antidepressives inhibit monoamine oxidase e.g. imipramine.

Thus, until the central effects of antidepressive drugs have been investigated fully and indeed, until a better understanding of the fundamental physiological and biochemical mechanisms involved in the functioning of the brain is reached, progress in the development of new drugs, of specific value in the treatment of depression, will be delayed. This therefore provides the mandate for pharmacological research in this field.

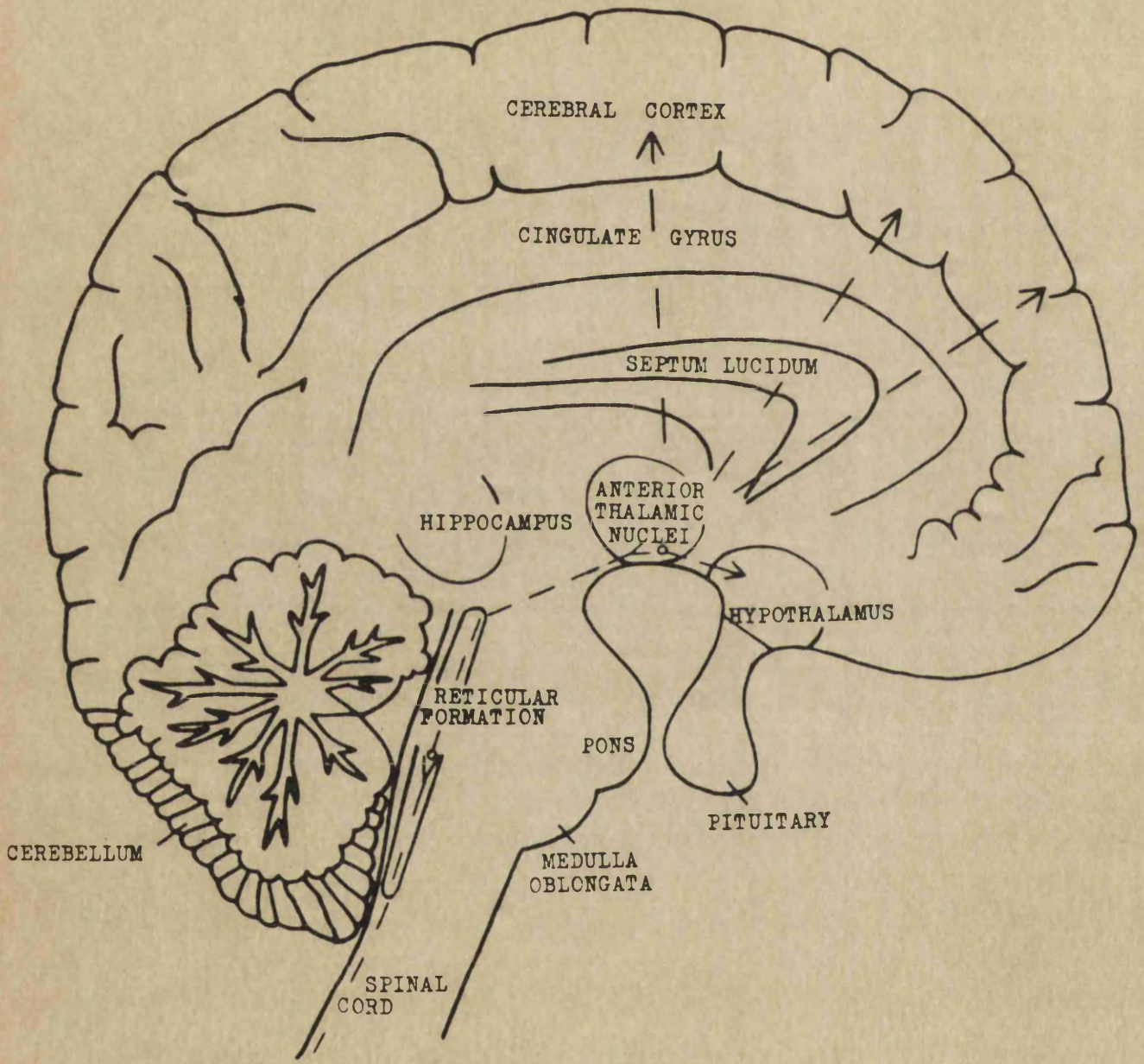

Fig. I.

Diagram of the Human Brain (Median Section). Showing the Position of the Reticular Formation in Relation to Other Structures with which it is Functionally Associated.

Theories on The Site and Mechanism of Action of Antidepressive Drugs.

Electrophysiological Aspects

The investigation of the effects of antidepressive drugs on the electrical activity of various functional systems of the brain, has provided valuable information concerning their central sites of action. In particular, three systems are thought to be primarily concerned in the activity of antidepressive drugs. These are, the reticular activating system, which consists of the reticular formation and the related thalamic nuclei, the thalamo-cortical system, consisting of the rostral portions of the ascending reticular activating system and a diffuse network having connections in the cortex, and the limbic system, which consists of the hippocampus, the cingulate gyrus and subcortical masses including the septum, the amygdala and certain diencephalic nuclei (Birzis, 1960) (Fig. I). Although the reticular activating and thalamo-cortical systems are involved generally in the central integration of motor and sensory impulses (Birzis, 1960), they are concerned more

specifically in the regulation of the sleep-wakefulness cycle (Moruzzi & Magoun, 1949; French, 1960; Killam, 1962). High frequency electrical stimulation of the midbrain reticular formation produces, in the sleeping or drowsy animal, signs of behavioural arousal and the appearance, in the cortical electroencephalogram (EEG), of fast low voltage waves (Killam, 1962). On the other hand, low frequency electrical stimulation of the non-specific thalamic nuclei, gives rise to the recruiting response which is characterised, in the EEG, by spindle bursts similar to those observed during barbiturate anaesthesia and similar to the resting (alpha) rhythm of the human EEG (Morrison & Dempsey, 1942; Birzis, 1960). While the limbic system is thought to be involved in emotional behaviour (Maclean, 1958), there is very little direct evidence that antidepressive drugs exert their characteristic pharmacodynamic effects by modifying this functional region. Indeed, the precise physiological significance of the limbic system, is, as yet, poorly understood (Birzis, 1960; Brady, 1960; Gloor, 1960; Green, 1960).

Amphetamine, which has been studied most

extensively with respect to its effects on the electrical activity of the brain (Toman & Davis, 1949; Himwich, 1959; Leake, 1958), produces, in the intact animal, symptoms of behavioural arousal and initiates a characteristically activated EEG pattern, consisting of fast low voltage waves (Longo & Silvestrini, 1957,a,b; Bradley & Elkes, 1957; Himwich, 1959; Killam, 1962). This alerting response is not abolished by high spinal section and indeed becomes more pronounced in the encéphale isolé preparation (Bradley & Elkes, 1957; Bremer & Stroupel, 1959; Shallek & Kuehn, 1959). When the influence of the reticular activating system is removed, either by section, as in the cerveau isolé preparation, or as a result of lesions of the midbrain reticular formation (Konigsmark, Killam & Killam, 1958; Killam, Gangloff, Konigsmark & Killam, 1959), the alerting response is prevented (Elkes, Elkes & Bradley, 1954). If, however, even small portions of the rostral reticular formation are allowed to remain, arousal can still be produced by the administration of suitable doses of amphetamine (Longo & Silvestrini, 1957,a). Further evidence that amphetamine has a direct effect

on the reticular activating system is provided by its ability to decrease the threshold for both behavioural and EEG arousal, initiated by electrical stimulation of the reticular formation (Monnier, 1957; Bradley & Key, 1958; Killam et al, 1959).

The relationship between the central pharmacodynamic effects of amphetamine and the electrical activity of the reticular formation, is even more complex than these experimental results suggest. Thus, although the EEG desynchrony induced by amphetamine can be abolished by the administration of atropine, behavioural arousal persists (Bradley, 1958). While the central electrophysiological activity of amphetamine has been attributed to a direct effect on adrenergic neurones in the reticular formation (Bradley & Elkes, 1957), it has been suggested (Himwich, 1959) that the ability of amphetamine to increase the effectiveness of sensory stimulation in causing EEG arousal (Rinaldi & Himwich, 1955; Bradley & Key, 1958), indicates that this drug increases the sensory input from the peripheral autonomic pathways (Himwich, 1959).

Since amphetamine is a sympathomimetic amine,

several investigations have been carried out to determine whether or not the EEG arousal response is related to a vascular change. Following the amphetamine-induced arousal of the cat encéphale isolé preparation, a marked decrease in the cerebrovascular resistance was observed (Ingvar, 1958). There is however, no evidence that this vascular effect initiates the alerting response (Birzis, 1960). Indeed, in the monkey, intracarotid administration of amphetamine has been reported to cause an increase in cerebrovascular tone (Dumke & Schmidt, 1943). In man, although amphetamine has been reported to cause a shift towards higher frequencies in the EEG (Gibbs & Maltby, 1943), it is generally thought to have very little effect on the electrical activity of the brain (Greville, Heppenstall, 1952), the total cerebral circulation or the cerebral metabolism, even in doses producing marked mental changes (Andrew & Sensenbach, 1955; Kety, 1959).

Although very little information is available concerning the central electrophysiological effects of the amphetamine-like drugs, methylphenidate and pipradrol, there is evidence that these compounds also cause

activation of the EEG. In experimental animals, methylphenidate produces effects similar to those of electrical stimulation of the reticular formation (Jouvet & Courjon, 1959). The EEG arousal thus initiated can be abolished by the destruction of the posterior diencephalon (Jouvet & Courjon, 1959). However, in man, therapeutic doses of methylphenidate produce no marked effects on the EEG (Pflanz & Schrader, 1959). Pipradrol increases the EEG alerting response caused by sensory and reticular-formation stimulation (Rinaldi & Himwich, 1955).

Caffeine also produces EEG arousal in the intact animal (Jouvet, Benoit, Marsallon & Courjon, 1957; Shallek & Kuehn, 1959; Krupp, Monnier & Stille, 1959). The increased frequency and low voltage activation of the EEG initiated by caffeine is not, however, prevented by removal of the influence of the reticular formation either by section or as a result of lesions of the diencephalic grey matter (Jouvet et al, 1957; Krupp et al, 1959). Indeed, the alerting effect has even been observed in the isolated cortical slab preparation (Jouvet et al, 1957). Caffeine has also been reported

to inhibit the cortical recruiting response to mediothalamic stimulation (Krupp et al, 1959), by a direct action on the diffuse thalamocortical circuit. Thus, while caffeine produces EEG arousal similar to that produced by amphetamine, there is no direct evidence that this drug exerts an effect on the reticular formation (Killam, 1962).

Dimethylaminoethanol, a central stimulant drug believed to be a precursor of acetylcholine (Murphree, Jenney & Pfieffer, 1959), has also been reported to initiate a weak alerting response in the EEG of the intact animal (Konigsmark et al, 1958). However, the results of experiments designed to compare the effects of this drug on the arousal patterns evoked by stimulation, either of peripheral nerves or various regions of the brain, including the mesencephalic reticular formation and the central thalamic nuclei, were equivocal. Whereas, in some animals, low doses induced spontaneous arousal and lowered the threshold at which EEG alerting could be initiated, in other animals, large doses were very often ineffective. This inconsistency has been interpreted as indicating the need

for the existence of a particular biochemical balance in order that dimethylaminoethanol might cause arousal of the cortical EEG (Killam, 1962). Although this drug may be a precursor of acetylcholine (Murphree et al, 1959), it has been suggested that when effective in producing EEG arousal, dimethylaminoethanol probably acts by stimulation of the reticular formation, thus resembling amphetamine (Killam, 1962). This hypothesis is compatible with the finding that both acetylcholine and adrenaline lower the threshold of the reticular formation to stimulation (French, 1960).

Although very large doses of iproniazid (500 to 900 mg/kg) have been reported to cause EEG arousal (Himwich, 1959), the daily administration of low doses (10 to 25 mg/kg) has been found to have no lasting effect on the EEG pattern (Shallek, 1960). Recent investigations of the central electrophysiological effects of the other monoamine oxidase inhibiting antidepressive drugs, tranylcypromine and nialamide, have revealed a gradual, daily, slowing of the EEG pattern which becomes similar to that recorded during sleep, even although the animals remain awake (Funderburk,

Finger, Drakontides & Schneider, 1962). These results are in marked contrast to those obtained by Costa and his colleagues (Costa, Pscheidt, Van Meter & Himwich, 1960), who found that pheniprazine and tranylcypromine produced an EEG activation, which, though slow in onset, was more prolonged than that initiated by amphetamine.

The iminodibenzyl derivative, imipramine, as well as causing depression of the reticular activating system, produces a slowing of the EEG which is characterized by the appearance of high amplitude waves and spindles (Bradley & Key, 1959; Birzis, 1960). The central electrophysiological effects of imipramine are therefore quite unlike those of the other antidepressives and indeed they resemble more closely the effects of the chemically related phenothiazine tranquillisers (Birzis, 1960).

Thus, although a number of antidepressive drugs have been found to produce similar effects on the EEG, it has so far been impossible, because of the lack of data, either to define their precise sites of action or to correlate pharmacodynamic activity with particular

effects on the electrical activity of various areas of the brain. While there is some evidence that amphetamine in particular and possibly also pipradrol and methylphenidate produce EEG arousal by modifying the activity of the reticular activating system (Killam, 1962), it is apparent from the investigations involving imipramine, that antidepressant activity is not associated with one particular effect in one specific area of the brain.

The Effects of Antidepressive Drugs on Central Synaptic Transmission.

The concept of neurohumoral transmission, first postulated to explain the propagation of nervous impulses at the endings of the postganglionic sympathetic fibres (Elliott, 1904) and later extended to include post-ganglionic parasympathetic (Loewi, 1921), ganglionic (Feldberg & Gaddum, 1934) and neuromuscular synapses (Dale, Feldberg & Vogt, 1936; Brown, Dale & Feldberg, 1936; Brown, 1937), has offered a basis for interpreting the pharmacodynamic activities of many peripherally acting drugs, e.g. atropine, hexamethonium and d-tubocurarine. Although confirmatory evidence exists (Brock, Coombs & Eccles, 1952; Eccles, Eccles & Fatt, 1956; Grundfest, 1957; Eccles & Jaeger, 1958; De Robertis, 1959; 1961), the application of this concept to central synapses (Dale, 1937) has not been unequivocally accepted (Crossland, 1960). The complexity of the brain has rendered difficult the identification of the chemical mediator or mediators involved in central synaptic transmission. Nevertheless, the pharmacological activities of a number of centrally acting drugs, including the antidepressives, have been

correlated with their ability to modify the metabolism of several biologically active compounds, postulated to have a transmitter function within the brain. These substances include, acetylcholine (MacIntosh, 1941; Feldberg & Vogt, 1948), adrenaline, noradrenaline (Vogt, 1954) and dopamine (Carlsson, 1959), 5-hydroxytryptamine (Amin, Crawford & Gaddum, 1954), gamma-aminobutyric acid (Berl & Waelsch,1958), histamine (Kwiatkowski, 1943), substance P (Von Euler & Gaddum, 1931; Hellauer & Umrath, 1948), adenosinetriphosphate (Holton & Holton, 1954; Holton, 1959) and the cerebellar factor of Crossland and Mitchell (1956).

Support for a central transmitter role for acetylcholine is particularly strong. Investigation of the synthesis (Feldberg et al, 1948; Hebb & Silver, 1956), distribution (MacIntosh, 1941) and hydrolysis (Aldridge & Johnson,. 1959) of acetylcholine in the brain and spinal cord, has revealed that while cholinesterase is widely distributed (Burgen & Chipman, 1951), both acetylcholine and choline acetylase are confined to certain areas. The occurrence, within the central nervous system, of alternating regions of high (e.g. the motor cortex,

thalamus and the anterior spinal roots) and low (e.g. the cerebellum, dorsal roots and the optic nerve) choline acetylase activity (Feldberg et al, 1948; Harris & Lin, 1951), has been interpreted as indicating the existence of both cholinergic and non-cholinergic neurones (Crossland, 1960; Robson & Stacey, 1962). Indirect evidence in favour of a central neurohumoral role for acetylcholine has been its discovery, using histochemical techniques, in brain homogenates, in association with the terminal portions of the axon and the presynaptic membrane (Whittaker, 1959; Gray & Whittaker, 1962). Further evidence for the participation of acetylcholine in central synaptic transmission is provided by the sensitivity of the Renshaw cells of the spinal cord to acetylcholine and anticholinesterase drugs and also by the depression of these cells caused by the acetylcholine antagonists atropine and β-erythroidine (Curtis & Eccles, 1958). Moreover, MacIntosh and Oborin (1953) demonstrated that during deep anaesthesia, the amount of acetylcholine which exuded from the exposed surface of the cortex was reduced. Indeed, when the electrical activity of the

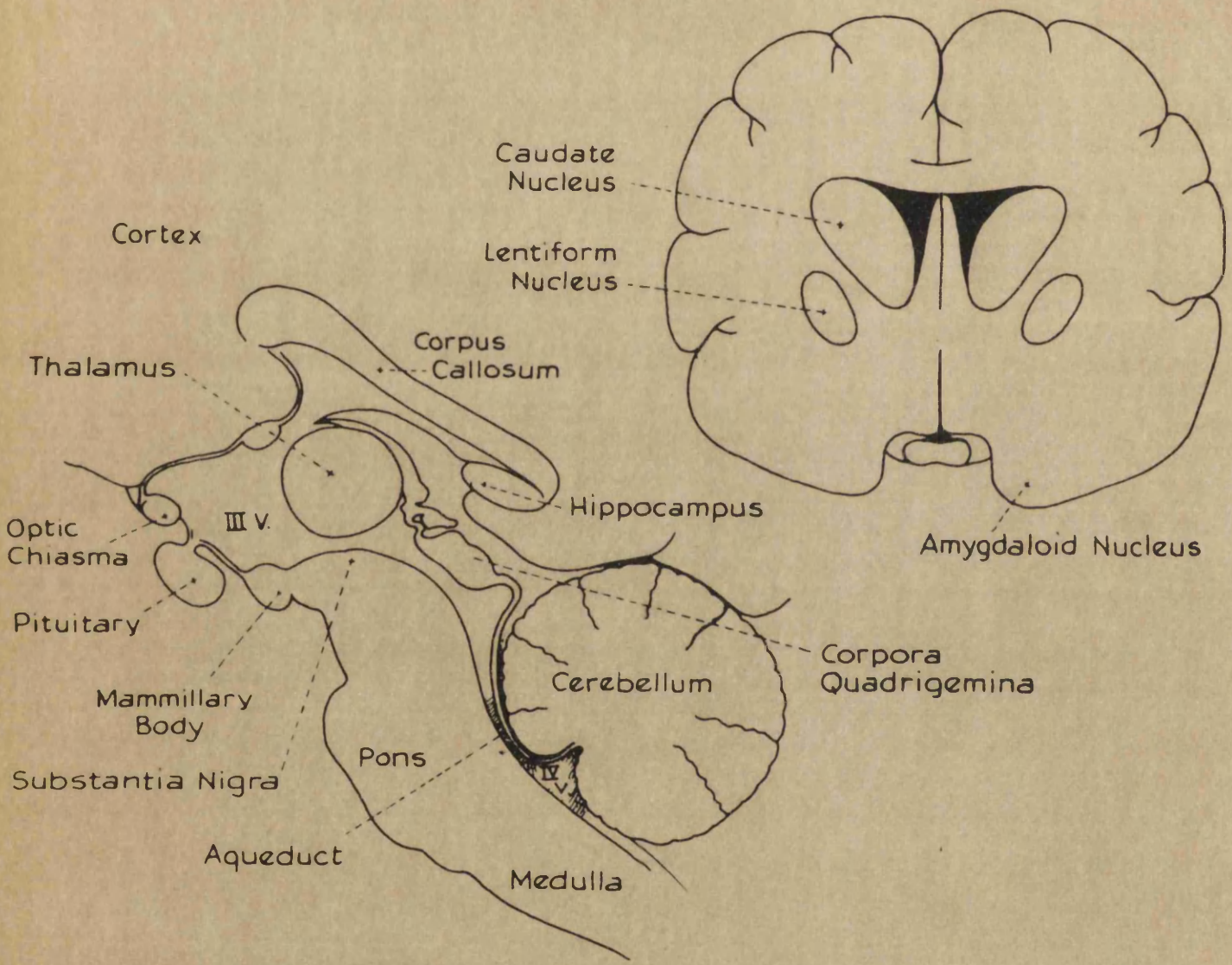

Fig. II

Diagram of a Generalised Mammalian Brain (Transverse Section and Medial Section in the Region of the Pons, Cerebellum and Midbrain). Showing the Areas which contain High Concentrations of a Number of Pharmacologically Important Amines (see also Figs. III, IV and V).

cortex was abolished completely, by undercutting it, the production of acetylcholine ceased altogether. Generally, it has been found that there is an inverse relationship between the amount of acetylcholine in the brain and the level of central nervous activity (Richter & Crossland, 1949; Crossland & Merrick, 1954).

Thus, although central nervous activity can be associated with changes in the levels of acetylcholine and although the synthesis, distribution and release of this compound, in the brain, are suggestive of a neurohumoral function, the evidence supporting this hypothesis is still less conclusive than that for cholinergic transmission in the peripheral nervous system, where despite the continuing controversy regarding its precise mechanism of action (Nachmansohn, 1959; Katz, 1962; Koelle, 1962), acetylcholine is universally believed to act as the chemical transmitter.

As a result of the existence in the central nervous system of non-cholinergic as well as cholinergic neurones, it has been suggested that the concept of chemical transmission demands at least one other transmitter (Perry, 1956). Interest in a central

NORADRENALINE

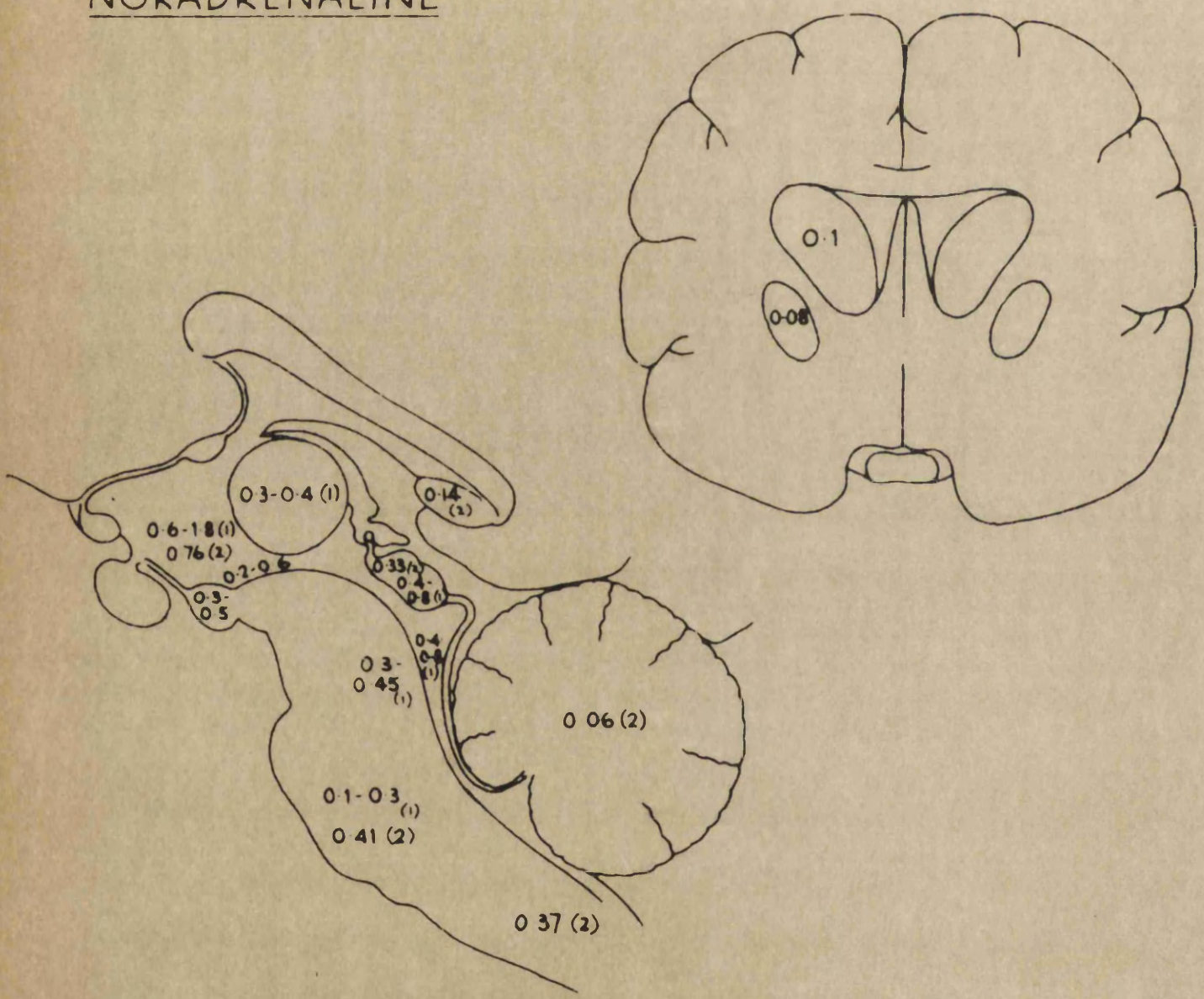

Fig. III

Diagram of a Generalised Mammalian Brain. Showing the concentrations of Noradrenaline in the Different Regions. The values are in Micrograms/G of Fresh Tissue. After (Vogt, 1954) (1) (Carlsson, 1959) (2).

transmitter role for catechol amines has been aroused by analogy with the peripheral nervous system where transmission at the endings of the postganglionic sympathetic fibres is effected by the release of a mixture of adrenaline and noradrenaline (Elliott, 1904; Cannon & Uridil, 1921; Von Euler, 1946) and where also adrenaline might act by modifying cholinergic transmission at the parasympathetic ganglion and the neuromuscular junction (Bülbring & Burn, 1939; Bülbring, 1944; Crossland, 1960).

The presence of adrenaline and noradrenaline in the brain was first demonstrated by Von Euler (1946) and later confirmed by Holtz (1950) who showed that brain sympathin contained a preponderance of noradrenaline. There are also present in the brain considerable quantities of dopamine, the immediate precursor of noradrenaline (Demis, Blaschko & Welch, 1956; Udenfriend & Wyngaarden, 1956). The investigation of the levels of catechol amines in the different parts of the brain has shown that although there are certain similarities in the central distribution of these compounds, there are also a number of important differences. The

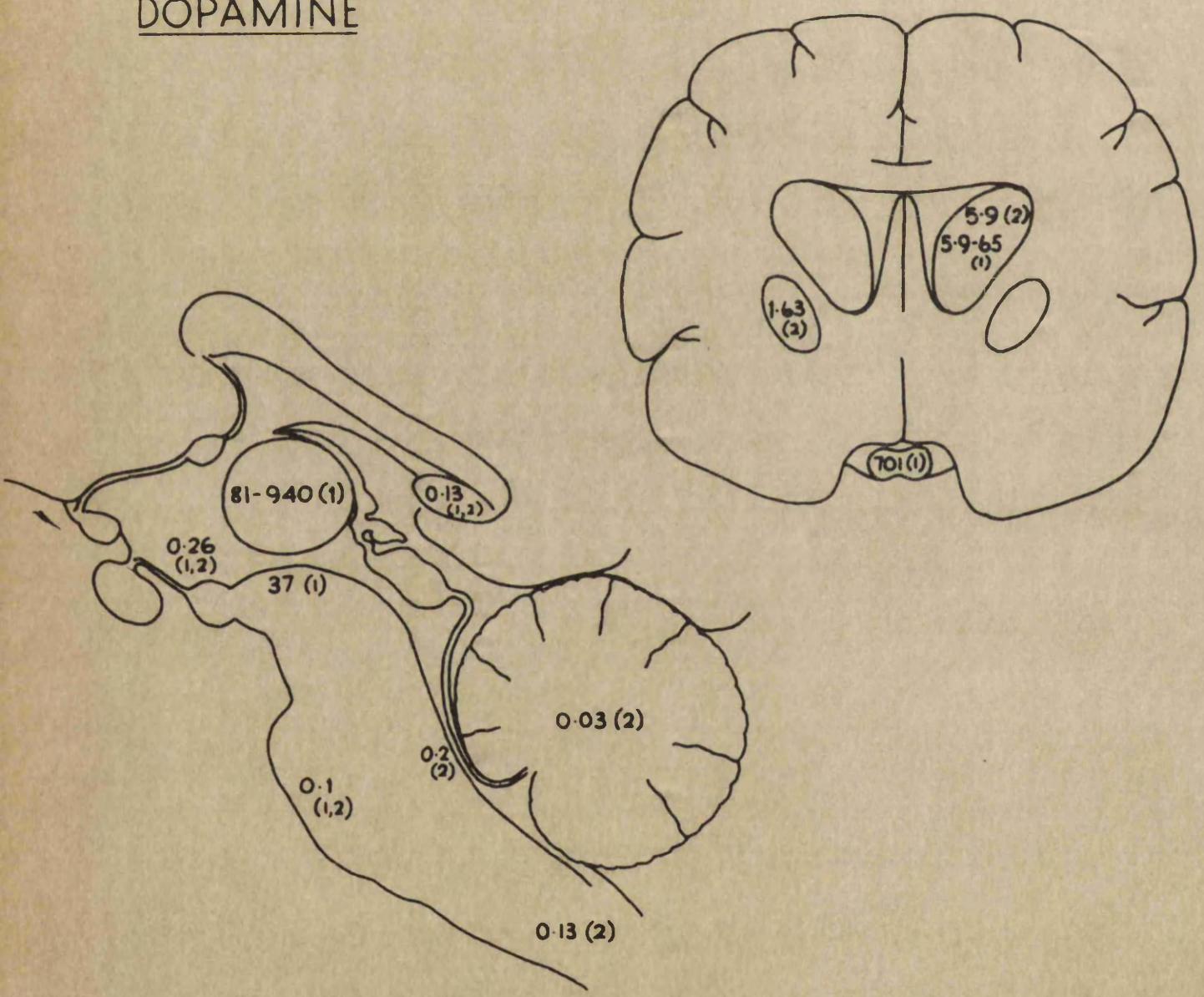

Fig. IV

Diagram of a Generalised Mammalian Brain.
Showing the concentrations of Dopamine in the Different Regions. The values are in Micrograms/G of Fresh Tissue. After (Garattini & Valzelli, 1962) (1), (Carlsson, 1959) (2).

highest concentrations of noradrenaline are in the hypothalamus, the reticular formation and the medial thalamic tract (Robson et al, 1962). While there are also high concentrations of dopamine in the hypothalamus, the largest amounts are in the caudate nucleus which contains very little noradrenaline (Vogt, 1954). Further indirect evidence in support of a central neurohumoral function for catechol amines, is provided by the presence within the brain of the enzymes necessary for their synthesis (DOPA decarboxylase) (Bogdanski, Weissbach & Udenfriend, 1957; Udenfriend & Creveling, 1959; Kuntzman, Shore, Bogdanski & Brodie, 1961) and inactivation (Monoamine oxidase and ortho-methyltransferase) (Bogdanski et al, 1957; Axelrod, Albers & Clemente, 1959).

The study and interpretation of the central actions of catechol amines are complicated both by their direct vascular effects (Schmidt, 1934a,b; Fog, 1939; Bonvallet, Dell & Hiebel, 1954) and by their virtual inability to pass the blood-brain barrier (Raab & Gigee, 1951; Schayer, 1951; Axelrod, Weil-Malherbe & Tomchick, 1959). However, when this difficulty was

overcome by direct injection of adrenaline into the lateral ventricles of the cat brain, it was found that, while small doses produced light anaesthesia (Feldberg & Sherwood, 1955), the effects on the electrical activity of the brain were marked by the activation of the EEG (Dell, Bonvallet & Hugelin, 1954), similar to that caused by electrical stimulation of the ascending reticular formation (Bonvallet et al, 1954; Dell et al, 1954; Porter, 1952).

Catechol amines produce a variety of effects on the brain. These include, the augmentation of both mono-and polysynaptic reflexes (Sigg, Ochs & Gerard, 1955), the reduction of evoked potentials of the visual cortex, produced, either by visual stimuli or by stimulation of the optic tract (Marrazzi, 1943), the inhibition of the release of the antidiuretic hormone, induced by intravenous injection of acetylcholine (Duke & Pickford, 1951) and the inhibition of the output of thyroid hormone (Von Euler, 1956). While adrenaline and noradrenaline can modify various aspects of brain function, the mechanisms by which these effects are achieved remain unexplained (Robson

5-HYDROXYTRYPTAMINE

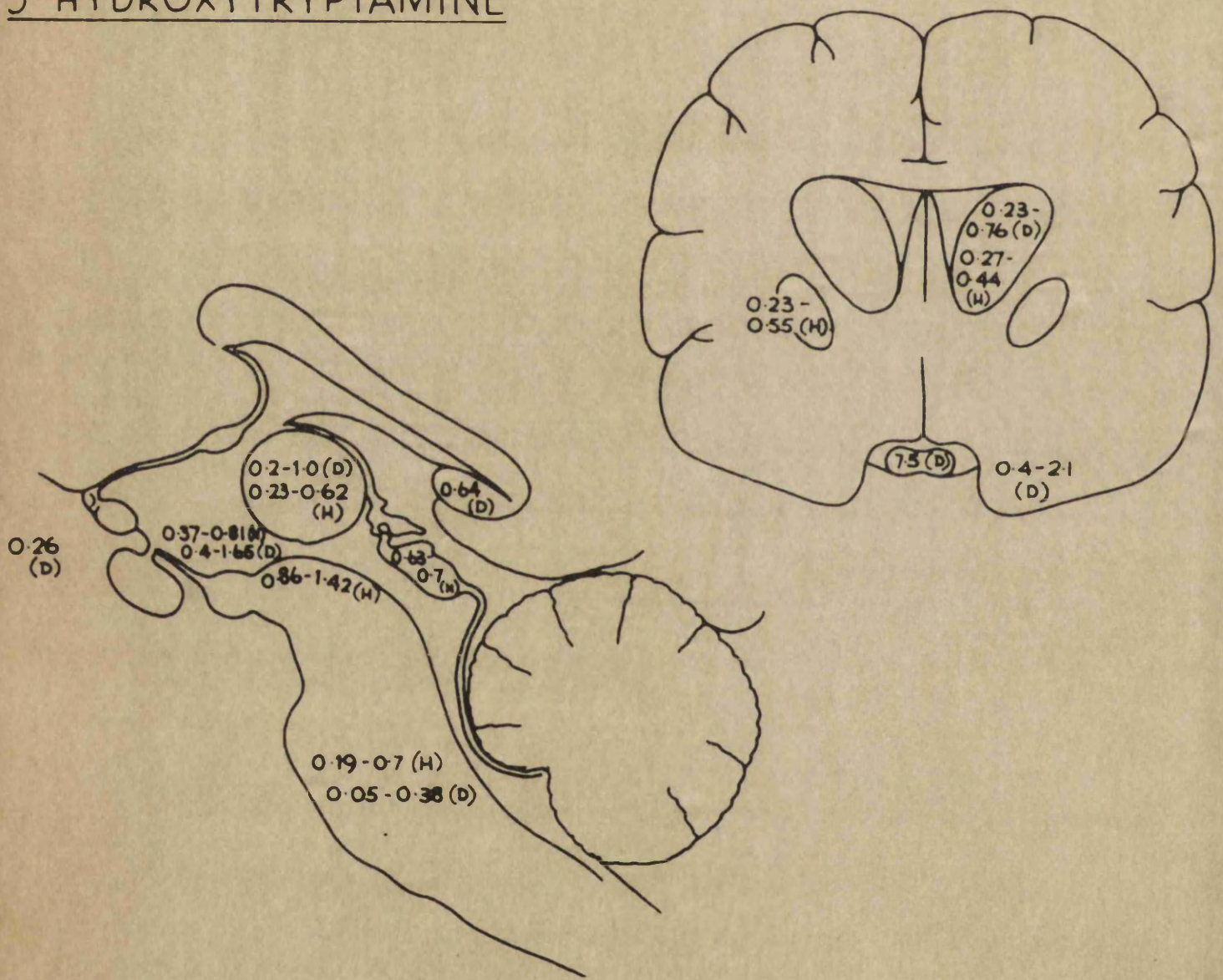

Fig. V.

Diagram of a Generalised Mammalian Brain (D, Dog; H, Human). Showing the concentrations of 5-Hydroxytryptamine in the Different Regions. The values are in Micrograms/G of Fresh Tissue. After (Garattini & Valzelli, 1962).

et al, 1962). Indeed, present evidence supporting a central neurohumoral function for either adrenaline, noradrenaline or dopamine is wholly indirect (Crossland, 1960). Despite the lack of supporting evidence it has been suggested (Brodie & Shore, 1957; Brodie, Spector & Shore, 1959) that noradrenaline might act as the central transmitter of the 'ergotrophic' (or central sympathetic) system, which is one of the two opposing nervous systems, postulated by Hess (1954) to explain the integration of central and peripheral autonomic function.

Brodie, Spector & Shore (1959) also suggested that the synaptic transmitter of the 'trophotrophic' (or central parasympathetic) system might be 5-hydroxytryptamine, a compound believed to be involved in brain functioning (Gaddum, 1953; Woolley & Shaw, 1954, 1957). The distribution of this compound and the enzyme ultimately responsible for its synthesis, 5-hydroxytryptophan decarboxylase, in areas of the brain such as the hypothalamus and the amygdala (Amin et al, 1954; Paasonen & Vogt, 1956), which are believed to be concerned in emotional behaviour (Crossland, 1960), might be

interpreted as indicating a relationship between 5-hydroxytryptamine and psychotropic drug action. When administered intraperitoneally to mice, 5-hydroxytryptamine causes sedation (Robson et al, 1962). It also causes both depression of the transcallosal cortical response (Marrazzi, 1957) and of the transmission of impulses evoked by stimulation of the optic nerve (Curtis & Davis, 1961). In contrast to this, the effects of 5-hydroxytryptamine on the electrocorticogram are characterised by desynchronization. While the EEG alerting response is thought to be caused by activation of the cortex and reticular formation, depression of the hypothalamic and thalamic nuclei has also been reported (Vogt, Gunn & Sawyer, 1957).

Although the rise in the brain levels of 5-hydroxytryptamine following the administration of its precursor, 5-hydroxytryptophan, was associated with signs of central stimulation (Udenfriend, Weissbach & Bogdanski, 1957), the increase in brain 5-hydroxytryptamine which followed the administration of either barbiturates, ether or a variety of anticonvulsants, was associated with cerebral depression (Bonnycastle, Giarman &

Paasonen, 1957; Anderson & Bonnycastle, 1960). These results are comparable with those obtained from experiments in which the effects of anticholinesterase drugs were studied on the behavioural and EEG patterns of the intact animal (Bradley & Elkes, 1957) and from other investigations in which the relationship between brain levels of acetylcholine and cerebral activity was studied (Crossland et al, 1949; Crossland et al, 1954).

Thus, although there is some evidence that 5-hydroxytryptamine is involved in the functioning of the brain, since the exact nature of its role has not been elucidated, it cannot as yet be assumed to act as a central transmitter.

While there is very little precise information available concerning the central distribution of both gamma-aminobutyric acid and the enzyme responsible for its synthesis, glutamic acid decarboxylase, it appears that in general the highest concentrations of these two substances in the brain are to be found in the grey matter (Elliott & Jasper, 1959). Interest in the activity of gamma-aminobutyric acid as a possible neurohormone has been provoked by the suggestion that

it might act as a transmitter of inhibitory impulses in the crustacean stretch receptor. In this preparation gamma-aminobutyric acid has a hyperpolarising effect on the membrane similar to that produced by stimulation of the inhibitory nerves (Kuffler & Edwards, 1958; Edwards & Kuffler, 1959). However, since the transmission of nervous impulses in invertebrates is very different from that in mammals (Robson *et al*, 1962), such evidence as this (Kuffler *et al*, 1958; Edwards *et al*, 1959) is only of limited value in understanding the central actions of gamma-aminobutyric acid (Curtis, 1961). Less evidence is available in support of a central neurohumoral function for either histamine, substance P, adenosinetriphosphate or the cerebellar factor (Crossland, 1960; Robson *et al*, 1962).

It may therefore be concluded that although there is strong evidence in favour of a chemical mechanism for central synaptic transmission, the chemical mediator or mediators involved have not yet been positively identified. While the most likely candidate is probably acetylcholine, other compounds, such as

noradrenaline and 5-hydroxytryptamine, are also of interest. Indeed, a great deal of the current research in this field is based upon the assumption that a number of antidepressive drugs produce their characteristic pharmacodynamic effects by interfering with the central metabolism of the hypothetical neurohormones, 5-hydroxytryptamine and noradrenaline.

Following their observation that amphetamine prevented the inhibition of respiration of brain cortex slices, caused by the addition to the system of either tyramine or tryptamine, Mann and Quastel (1940) suggested that the central stimulant activity of this drug might be related to its ability to compete with these amines for monoamine oxidase, thereby reducing the rate of formation of the inhibitory aldehydes, produced as a result of amine metabolism, according to the reaction.

$$R - CH_2\overset{+}{N}HR^1R^{11} + O_2 \xrightarrow[MAO]{} RCHO + NH_2R^1R^{11} + H_2O_2$$

(Jacobsen, 1959). However, more recent investigations have shown this hypothesis to be unsatisfactory, since

no correlation exists between the central stimulant activity of the separate stereoisomers of either amphetamine or 1-phenylethylamine and their ability to inhibit monoamine oxidase (Grana & Lilla, 1959). When amphetamine was administered in doses sufficient to cause marked central stimulation, in vivo monoamine oxidase activity was unaffected (Schayer, 1953; Sjoerdsma, Gillespie & Udenfriend, 1959; Paasonen & Vogt, 1956). Indeed, amphetamine has been reported to produce a decrease in the brain levels of noradrenaline (McLean & McCartney, 1961; Sanan & Vogt, 1962; Baird & Lewis, 1963), an effect which might be caused by the inhibition of dopamine-β-oxidase, the enzyme thought to be involved in the conversion of dopamine to noradrenaline (Goldstein & Contrera, 1961).

While, however, the central stimulant activity of amphetamine cannot be related to the inhibition of monoamine oxidase, this concept has since been reintroduced to explain the pharmacological effects of another group of compounds, having both central stimulant and in vitro and in vivo monoamine oxidase inhibitory activity (Zeller & Barsky, 1952; Brodie

et al, 1957; Brodie et al, 1959; Costa, Gessa, Hirsch, Kuntzman & Brodie, 1962). Thus, it has been suggested that the central activity of drugs such as iproniazid and other hydrazine derivatives, is related to their ability to increase the levels of amines in the brain. This hypothesis is supported by the fact that the administration to animals of 5-hydroxytryptophan or 3,4-dihydroxyphenylalanine, which are respectively the precursors of 5-hydroxytryptamine and dopamine, produces signs of behavioural excitation as well as increasing the brain levels of these amines (Bertler & Rosengren, 1959; Bogdanski, Weissbach & Udenfriend, 1958; Costa & Rinaldi, 1958). Furthermore, it has been reported that when iproniazid is administered together with 5-hydroxytryptophan or 3,4-dihydroxy-phenylalanine, the degree of behavioural stimulation produced, is greater than when either of the amino acids is given alone (Mitoma, 1960). However, the relationship between monoamine oxidase blockade and central stimulatory activity is not a simple one and indeed, many of the experimental results indicate basic weaknesses in the hypothesis. In particular,

the evidence concerning the amine or amines involved in mediating the pharmacodynamic effects of these drugs, is contradictory. The central effects of iproniazid have variously been related to an increase in the brain levels of noradrenaline in the rabbit (Spector, Shore & Brodie, 1960) and to an increase in the brain levels of 5-hydroxytryptamine in the cat (Funderburk, Finger, Drakontides & Schneider, 1962). It has also been reported that in rats the elevation in the brain levels of noradrenaline, following treatment with iproniazid, is not associated with any signs of central stimulation (Green & Sawyer, 1960). Carlsson has further suggested (1960) that the central stimulatory activity of monoamine oxidase inhibiting drugs is more closely related to their ability to increase the brain levels of normetadrenaline, rather than noradrenaline or 5-hydroxytryptamine. The major difficulty in correlating central stimulant activity with changes in the brain levels of specific amines arises from the ability of the enzyme monoamine oxidase to oxidise a number of amines, including adrenaline, noradrenaline, dopamine, tryptamine, 5-hydroxytryptamine

and tyramine (Tabor, Tabor & Rosenthal, 1954; Imaizumi, Omori, Unoki, Sano, Watari, Namba & Inui, 1959; Weissbach, Redfield & Udenfriend, 1957; Pratesi & Blaschko, 1959). Another complicating factor to be considered, is that the enzyme ortho-methyl transferase may also be concerned in the metabolism of noradrenaline (Axelrod, 1960).

Other experiments have shown that even when the monoamine oxidase activity of cat or mouse brain was completely inhibited by nialamide (Funderburk et al, 1962), phenelzine or pheniprazine (Dubnick, Leeson & Phillips, 1962), the administration of higher doses of these drugs brought about a further elevation of the brain levels of noradrenaline and 5-hydroxytryptamine. This indicates that the increase in brain levels of these amines is probably due, at least in part, to a mechanism other than monoamine oxidase inhibition. A possible explanation of this mechanism might be that drugs such as iproniazid and phenelzine prevent the release of amines from their binding sites within the brain (Pletscher, 1956; Giarman & Schanberg, 1958; Axelrod, 1962). Some

support for this hypothesis is provided by the observation that iproniazid prevents the reserpine-induced release of 5-hydroxytryptamine from blood platelets *in vitro* (Paasonen & Pletscher, 1960).

More recently, the central excitation produced by monoamine oxidase inhibiting drugs has been directly attributed to an elevation of the brain levels of free noradrenaline, a response believed to be associated with ergotrophic predominance (Costa *et al*, 1962). While this hypothesis has been valuable in interpreting the central effects of these drugs, it has not been generally accepted (Sanan *et al*, 1962).

Imipramine, though not a potent inhibitor of monoamine oxidase (Pulver, Exer & Herrmann, 1960), produces a highly significant increase in the brain levels of 5-hydroxytryptamine (Costa, Garattini & Valzelli, 1960). It also causes a reduction in the *in vivo* 5-hydroxytryptamine content of blood platelets in humans (Marshall, Stirling, Tait & Todrick, 1960). While these observations confirm the view that central excitation can be associated with an effect on amine metabolism, the exact nature of this relationship

remains unexplained.

Although the evidence in favour of a central transmitter function for acetylcholine is much more convincing than that for noradrenaline or 5-hydroxytryptamine, fewer attempts have been made to correlate the effects of centrally acting drugs with the modification of central acetylcholine metabolism. However, since imipramine has some anticholinergic activity (Domenjoz & Theobald, 1959) and since a number of anticholinergic compounds have antidepressive activity (Fink, 1958, 1960; Abood, Ostfield & Biel, 1958, 1959), it has been postulated that antidepressive activity might be associated with atropine-like activity (Biel, Nuhfer, Hoya, Leiser & Abood, 1962). On the basis of an extensive pharmacological investigation, in which it was found that among a series of acetic acid esters of heterocyclic amino alcohols, only compounds with atropine-like activity were central nervous system stimulants, Biel and his colleagues (1962) suggested that anticholinergic compounds might act by interfering with the action of acetylcholine in the trophotrophic

system. However, this view is contradicted both by the electrophysiological (Killam, 1962) and clinical evidence relating to the central effects of other anticholinergic drugs. Although atropine itself produces central excitation at toxic dose levels and although hyoscine can cause similar effects in elderly patients, neither of these potent anticholinergic drugs can be regarded as a central nervous system stimulant.. Indeed, while atropine has little central activity at therapeutic doses, hyoscine produces sedation and marked central nervous depression.

On the other hand, it has been postulated that central stimulant activity might be associated with central cholinergic activity. Thus, the central stimulant, dimethylamino-ethanol has been suggested to act as an acetylcholine precursor within the brain (Murphree et al, 1959). Although this view is supported by the electrophysiological data relating to the EEG arousal induced by anticholinesterase drugs, the lack of correlation existing between these effects and those on the gross behaviour of the animal,

constitutes a serious weakness in this hypothesis. Nevertheless, imipramine has been reported to have anticholinesterase activity (Pulver et al, 1960). Whether or not this property can be associated with antidepressive activity is a problem requiring further investigation.

Apart from a number of isolated observations, such as that concerning the rise in the brain levels of gamma-aminobutyric acid, following treatment with iproniazid and other monoamine oxidase inhibiting compounds (Elliott & Van Gelder, 1960), the effects of central stimulant drugs on the metabolism of other possible central synaptic transmitters have received little attention. However, until the precise function within the brain of gamma-aminobutyric acid, substance P, 5-hydroxytryptamine and indeed, even noradrenaline and acetylcholine, have been established, theories seeking to explain the mechanism of action of central stimulant drugs on the basis of the modification of the metabolism of a particular substance, will be unsatisfactory and open to criticism.

The Effects of Centrally-Acting Drugs on the Energy Metabolism of the Brain.

A number of attempts have been made to explain the pharmacodynamic effects of central nervous system depressants, on the basis of an ability to reduce the amount of energy available for the functioning of the brain (Butler, 1950; Quastel, 1952, 1955; McIlwain, 1959, 1962; Aldridge, 1962). Ether, chloral and the barbiturates have long been known to decrease the *in vitro* oxygen uptake of preparations of mammalian brain and to inhibit the *in vitro* oxidation of a variety of substrates, including glucose, pyruvate and lactate (Quastel & Wheatley, 1932). More recent studies have shown that the newer central nervous system depressants, chlorpromazine (Finkelstein, Spencer & Ridgeway, 1954) and reserpine (Century & Horwitt, 1956) also depress *in vitro* brain respiration. These observations are, however, of limited value in explaining the mechanisms of action of centrally-acting drugs, since the concentrations required to produce *in vitro* inhibition of respiration, are far greater than those likely to be found in the brain, *in vivo*,

following the administration of therapeutic doses of these drugs (McIlwain, 1953, 1962). Indeed, while low concentrations of barbiturates, sufficient to cause *in vivo* depression of brain functioning, have no effect on brain respiration *in vitro* (McIlwain, 1953; Cohen & Heald, 1960), low concentrations of ethanol (Wallgren & Kulonen, 1960) and phenobarbitone (Westfall, 1949) have even been reported to produce slight stimulation of *in vitro* oxygen uptake.

A number of techniques have been employed to increase the rate of oxygen uptake of brain preparations *in vitro*, in order to produce a level of respiration, similar to that found *in vivo*. When the oxygen uptake was increased, by means of electrical stimulation, the concentrations of chloral, urethane, chlorpromazine and barbiturates, required to inhibit respiration, were comparable with those which might be found in the brain *in vivo*, following the administration of therapeutic doses of these drugs (McIlwain, 1953; Forda & McIlwain, 1953; McIlwain & Greengard, 1957). Similar effects have been reported for ethanol, chloretone and barbiturates, when the respiration was stimulated

by potassium ions (Ghosh & Quastel, 1954; Geddes & Quastel, 1956) and for chlorpromazine, when the oxygen uptake was stimulated by 2,4-dinitrophenol (Kok, 1956).

The interpretation of these observations is, however, made difficult by the contradictory nature of the results of *in vivo* investigations. Thus, high doses of chlorpromazine, for example, have been found to have no effect on brain respiration, both in man (Fazekas, Albert & Alman, 1955) and in the dog (Frowein, Hirsch, Kayser & Krenkel, 1955). This absence of effect may be compared with the unchanged respiration of the brain during sleep or mild sedation (McIlwain, 1962) and may be contrasted with the marked inhibition of brain oxygen uptake, observed during general anaesthesia (Quastel, 1952). In general, while the pharmacological activity of chlorpromazine cannot be correlated with an effect on brain respiration, unless a selective concentration at specific sites within the brain is assumed (Hunter & Lowry, 1956; Lewis, 1963), the central nervous depression, caused by anaesthetic doses of ethanol, ether, barbiturates and

other central nervous system depressants, does appear to be associated with an inhibition of oxygen uptake (Kety, 1948; Wechsler, Dripps & Kety, 1951; Fazekas & Bessman, 1953; Gordon, 1956; Schmidt, Kety & Pennes, 1945). Whether this response is the result of generalised central nervous system depression or whether it is in fact the cause, has not been established (Hunter & Lowry, 1956).

Fewer attempts have been made to explain the pharmacodynamic activity of central nervous system stimulant drugs, on the basis of an effect on brain energy metabolism. Nevertheless, amphetamine, at high dose levels, inhibits the in vitro oxygen uptake of brain slices (Mann et al, 1940; Lu & Krantz, 1953). On the other hand, while caffeine was found to increase the rate of brain respiration in vitro (Levy, 1946), iproniazid was found to have no consistent effect (Weiner, 1959). Central nervous stimulant drugs have also been reported to have no marked effect on in vivo brain respiration (Shenkin, 1951; Kety, 1959). These investigations have thus provided very little evidence to indicate the existence of a direct

relationship between central nervous system stimulant activity and the modification of brain energy metabolism.

The effects of centrally-acting drugs on other aspects of brain energy metabolism have been investigated in an effort to determine their primary biochemical sites of action. A number of central nervous depressant drugs have been postulated to act by interfering with the synthesis of adenosine-triphosphate (Quastel, 1952, 1955). Since the brain obtains most of the energy necessary for its functioning from the hydrolysis of adenosinetriphosphate, a reduction in the yield of this compound might explain the mechanism of action of some central nervous depressant drugs. This hypothesis is supported by the fact that a variety of depressant drugs, including a steroidal anaesthetic (Truit, Bell & Krantz, 1956), the barbiturates, pentobarbitone and amylobarbitone (Brody & Bain, 1951), chlorpromazine (Abood, 1955) and reserpine (Abood & Romanchek, 1957) uncouple oxidative phosphorylation, as shown by their ability to reduce the ratio of phosphate to oxygen uptake, in vitro, in preparations of brain slices and in mitochondria.

Not all central nervous system depressants uncouple oxidative phosphorylation. Thus, while phenobarbitone and allobarbitone (Brody & Bain, 1954; Wolpert, Truit, Bell & Krantz, 1956) inhibit both oxygen and phosphate uptake almost equally, ethanol, chloral, paraldehyde and morphine have no effect on the ratio of phosphate to oxygen uptake in brain mitochondria (Wolpert et al, 1956; Levy & Featherstone, 1954). Furthermore, the potent uncoupling agent, 2,4-dinitrophenol does not have any central nervous system depressant activity (Butler, 1962). The ability to uncouple oxidation from phosphorylation does not therefore appear to be a property common to all central nervous system depressant drugs.

In vivo investigations have confirmed that the decreased oxygen uptake found during general anaesthesia is not associated with a reduction in the adenosinetriphosphate content of the brain (Stone, 1940; Le Page, 1946; Lin, Cohen & Cohen, 1958; Gerlach, Doring & Fleckenstein, 1958). These results therefore appear to indicate that the synthesis of

adenosinetriphosphate is not impaired by central nervous system depressant drugs. However, there is some disagreement as to whether the tranquillising drugs, chlorpromazine and reserpine, do in fact interfere with this mechanism. Thus, while Grenell, Mendelson and McElroy (1955) found that chlorpromazine produced a rise in the brain levels of adenosine-triphosphate, Weiner and Huls (1961) and Minard and Davis (1962) found no alteration in the brain levels of either adenosinetriphosphate, adenosinediphosphate or phosphocreatine. On the other hand, phenobarbitone (Heim & Estler, 1961) and reserpine (Kirpekar & Lewis, 1959; Kaul & Lewis, 1963) have been found to cause a fall in the brain levels of adenosinetriphosphate. While these results might be interpreted on the basis of a decrease in the synthesis of this compound, they must be compared with those of Bain (1957) who showed that the incorporation of radioactive phosphate in adenosinetriphosphate was in fact greater in animals pretreated with anaesthetic doses of amylobarbitone and thiopentone, than in control animals. Whereas the pharmacodynamic activity of barbiturates seems to be

unrelated to their ability to inhibit the biochemical mechanisms involved in the production of adenosinetriphosphate and can probably best be explained on the basis of a decrease in its utilisation, the central nervous system depressant activity of reserpine and chlorpromazine may be due to a decreased energy production in one particular area of the brain (Quastel & Wheatley, 1932; Quastel, 1952; Van Petten, 1962).

Some correlation between central stimulant activity and the modification of brain phosphate metabolism has also been shown to exist. Thus, methyl amphetamine has been demonstrated to increase, in the rabbit, the brain levels of adenosinetriphosphate (Palladin, 1952; Palladin, Khaikina & Polyakova, 1952). A wide variety of central stimulants and related compounds including caffeine (Misson-Crighel, Constantinescu & Crighel, 1959), amphetamine, ephedrine, phenmetrazine, iproniazid, amitriptyline, imipramine and orphenadrine (Van Petten & Lewis, 1962; Lewis & Van Petten, 1962; Lewis & Van Petten, 1963) have also been shown to produce a rise in the brain levels of adenosinetriphosphate (ATP) in the rat and in

conjunction with this, a fall in the level of adenosinediphosphate (ADP), thereby increasing the ratio ATP/ADP, which may be taken as an index representing the balance between resynthesis and utilisation (Lewis & Van Petten, 1962). Similar effects have been reported in the rat, following treatment with the psychotomimetics, lysergic acid diethylamide, mescaline, bufotenine and psilocybin (Lewis, Ritchie & Van Petten, 1963) and in mice, after treatment with ether (Estler & Heim, 1960). Lewis and Van Petten have suggested tentatively (1962, 1963) that these results indicate a net increase in the resynthesis of adenosinetriphosphate. The adenosinetriphosphate thus made available may then be used for the synthesis of transmitters and the maintainance of ionic gradients within the brain, effects which might be manifested in the intact animal by the appearance of increased behavioural alertness (Lewis, 1963). In contrast with these results it has also been reported that the amount of adenosine-triphosphate in the brain is decreased by treatment with caffeine (Shapot, 1957) and pheniprazine

(Bernsohn, Possley & Custod, 1959). These observations are in agreement with the results of other experiments in which it was shown that in animals rendered hyperactive by the application of external stimuli, consisting of electric shocks or the rotation of the animals in a drum, the brain content of adenosinetriphosphate was reduced (Le Page, 1946; Sytinsky, 1956; Shapot, 1957). However, these apparently divergent results are not incompatible, since a reduction in the breakdown of brain adenosinetriphosphate is unlikely in alerted animals and since central excitation is in fact associated with a fall in the brain adenosinetriphosphate content (Le Page, 1946), the raised brain adenosinetriphosphate levels following treatment with central nervous system stimulants may indeed be an indication of an increase in its resynthesis.

Purpose of Research

None of the hypotheses postulated to explain the pharmacodynamic activity of central nervous stimulant drugs is entirely satisfactory. Attempts to relate their activity to a modification of the metabolism of possible brain neurohormones and to an effect upon brain energy metabolism are based on experimental evidence which is inconclusive and contradictory. It is, however, probable that most drugs having an effect upon the higher functions of the central nervous system, have at least indirect actions upon the enzymic activity of the brain. An attempt has therefore been made, in this investigation, to determine and compare the *in vitro* effects of a number of central nervous system stimulants and related compounds on the oxygen uptake of the brain and on the activity of several important enzymes, involved in brain functioning. The enzymes chosen for this present study were acetylcholinesterase, monoamine oxidase and adenosinetriphosphatase, all of

which are involved in the metabolism of compounds suggested to have an important function within the central nervous system (Robson & Stacey, 1962). While this investigation has been carried out, generally, in an attempt to provide information of value in understanding the basic mechanisms of action of central nervous stimulant drugs, it is concerned more particularly, with obtaining evidence which would permit a critical evaluation to be made of the present concepts relating to the pharmacodynamic activity of central nervous system stimulant drugs.

METHODS

METHODS

In this study, the investigation was limited to determining the effects of some centrally acting drugs on the *in vitro* enzymic activity of several preparations of rat brain, namely cerebral cortex slices, whole brain homogenates and mitochondria. The experimental methods used, involved manometric measurements of oxygen uptake and carbon dioxide production and spectrophotometric estimation of inorganic orthophosphate.

Male albino rats weighing from 150 to 200 g were used in all experiments. In order to avoid undue damage to the brain and to prevent bleeding into the skull during dissection, each animal was first stunned by means of a light blow delivered immediately behind the head before being killed by exsanguination. After removal of the skin covering the cranium, the top of the skull was carefully cut away. The whole brain was then excised and transferred to a suitable solution e.g. ice-cold, isotonic sucrose (0.25 M). Throughout these experiments the weighing of all tissues was carried out using a torsion balance.

Determination of the Oxygen Uptake of Cerebral Cortex Slices

Using the Warburg 'direct' method (Umbreit, Burris & Stauffer, 1957a), the effects of some centrally acting drugs were determined on the *in vitro* oxygen uptake of rat brain cerebral cortex slices. Rat brains were excised and transferred to ice-cold phosphate medium (Umbreit, Burris & Stauffer, 1957b). The cerebral cortex from each brain was dissected free and placed on a pad consisting of four hardened filter papers, moistened with cold phosphate medium. The tissue was cut to give sections of thickness 0.36 mm using a McIlwain tissue chopper (McIlwain & Buddle, 1953).

To each of 12 flasks (6 control and 6 drug treated), were added 2.4 ml of Krebs-Ringer phosphate (Umbreit, Burris & Stauffer, 1957b) containing 0.2 % glucose and 100 mg of tissue slices. The tissue was dispersed throughout the suspending medium by careful rotation of the flask. The centre well contained 0.2 ml of potassium hydroxide solution (20 % W/V) and a fluted alkali-resistant filter paper. The sidearm contained 0.4 ml of drug or control solution. After temperature equilibration (10 minutes) the stopcocks were closed

and oxygen uptake was measured for 1 hour in an atmosphere of air. The contents of the sidearm were then tipped into the main compartment and thereafter oxygen uptake was recorded at hourly intervals for 4 hours.

Determination of the Stimulated Oxygen Uptake of Cerebral Cortex Slices.

The respiration of rat brain cerebral cortex slices, stimulated in vitro by 2,4-dinitrophenol, was determined manometrically, the experimental procedure being the same as that described for normal respiration (page 48). In addition to phosphate medium and tissue slices, each flask contained in the main chamber, 2,4-dinitrophenol in a final concentration of 2.5×10^{-5} M (Kok, 1956). The drug and control solutions were tipped into the main chamber immediately before incubation. Oxygen uptake was then measured for 3 hours.

Determination of the Monoamine Oxidase Activity of Mitochondria.

The in vitro inhibition of rat brain mitochondrial monoamine oxidase was determined manometrically by a modification of the methods of Davison (1957) and

Randall and Bagdon (1959). Rat brains were excised, chilled in ice-cold isotonic (0.25 M) sucrose and weighed. A 10 % (W/V) homogenate was prepared in sucrose (0.25 M, 0°C). The tissue was disintegrated (2 min, 1500 rev/min, 0°C) in a Potter-Elvehjem-type homogeniser having a difference in radii between the Perspex pestle and the glass tube of 0.15 mm (McIlwain & Rodnight, 1962a). During homogenisation, the suspension was forced past the pestle and the walls of the tube 20 times per minute in each direction. Differential centrifugation was carried out to separate the mitochondrial fraction (McIlwain & Rodnight, 1962b). The homogenate was first centrifuged (10 min, 1000 x g, 0°C) to sediment the nucleii and cellular debris. After pipetting off the supernatant, the sediment was washed twice by rehomogenising (2 min, 1500 rev/min, 0°C) in sucrose (0.25 M) and recentrifuging (10 min, 1000 x g, 0°C). The supernatant and washings from the nuclear fraction were combined and centrifuged (15 min, 20,000 x g, 0°C) to sediment the mitochondria,which were then washed twice by resuspension in sucrose (0.25 M, 0°C) and recentrifuging (15 min, 20,000 x g, 0°C). Finally the

mitochondria were suspended in Sorensen's phosphate buffer (0.067 M, pH 7.2, 0°C) (Davison, 1957) so that 1.0 ml of suspension was equivalent to 500 mg (wet weight) of whole brain tissue.

The measurement of oxygen uptake was carried out in standard Warburg flasks at 38°C in an atmosphere of air. Each flask contained in the main compartment 1.0 ml of mitochondrial suspension as a source of enzyme and sodium cyanide, in a final concentration of 0.0033 M, to restrict the enzymic activity to that of monoamine oxidase, The drugs under investigation were dissolved in phosphate buffer (0.067 M, pH 7.2) and added to the main compartment 15 minutes prior to incubation. In all but two of the flasks, tyramine, in a final concentration of 0.01 M, was used as a substrate. A correction for enzymic activity other than that of monoamine oxidase was made by subtracting the mean of the two blank determinations from each of the values obtained for drug and control. The final volumes were adjusted to 3.0 ml with phosphate buffer (0.067 M, pH 7.2). After equilibration for 10 minutes the substrate was tipped into the main compartment and

oxygen uptake was recorded for 1 hour. The inhibition of oxygen uptake was taken as a measure of the monoamine oxidase inhibitory activity of each drug. Different molar concentrations of each drug were used, the mean of four observations (µl oxygen absorbed/500 mg (wet weight) of brain tissue/hr) at each dose level being calculated as a percentage inhibition of the control mean. The PI_{50} value, which is the negative logarithm of the molar concentration of drug producing an inhibition of 50 %, was then calculated according to the method of Blashko, Bülbring and Chou (1949)

Determination of the Acetylcholinesterase Activity of a Preparation of Whole Brain.

Using the method of Fenwick, Barron and Watson (1957), the in vitro inhibition of a preparation of rat brain acetylcholinesterase was determined manometrically by a modification of the technique of Ammon (1933). Rat brains were excised, rinsed free from blood in isotonic saline (0.9 % W/V sodium chloride) and weighed. The brains were disintegrated (2 min, 1500 rev/min, room temperature) in deionised water

using a homogeniser with a moderately loose fitting pestle. The homogenate, diluted with deionised water to produce a 5 % W/V suspension, was then maintained at room temperature for 2 hours. After centrifugation (10 min, 600 x g, room temperature), the supernatant was removed and used as a source of acetylcholinesterase activity.

Each flask contained 2.0 ml of Krebs-Ringer bicarbonate solution (Umbreit, Burris & Stauffer, 1957b), 0.25 ml of enzyme preparation in the main compartment and 0.5 ml of acetylcholine chloride solution (0.063 M) in the sidearm. Drugs under investigation dissolved in isotonic saline (0.9 % W/V sodium chloride), were added to the main compartment. In order to minimise the effects of anaerobic respiration involving endogenous substrates, the flasks were incubated for 30 minutes with the stopcocks open, before the acetylcholine was tipped into the main compartment. During the last 15 minutes of this preliminary period, the flasks were gassed with nitrogen. Measurements were then made of the volumes of carbon dioxide evolved in 10 minutes from the bicarbonate medium by acetic acid, produced as a result of the

enzymic hydrolysis of acetylcholine.

Blank determinations, omitting substrate, were carried out to estimate the carbon dioxide liberated from the medium by anaerobically produced lactic acid. The residual acetylcholinesterase activity in each flask was then calculated. The inhibition of the evolution of carbon dioxide was used as a measure of the anti-acetylcholinesterase activity of each drug. The mean inhibition at each dose level was calculated as a percentage of the control mean and plotted against the molar concentration. The PI_{50} value for each compound was then determined (Blashko, Bülbring & Chou, 1949)

Determination of the Adenosinetriphosphatase Activity of Whole Brain Homogenate.

Using a modification of the method of Lardy and Wellman (1953), the in vitro adenosinetriphosphatase activity of rat whole brain homogenate was determined in the presence of Ca^{++} and Mg^{++} ions (Potter, Siekevitz & Simonson, 1953). Rat brains were excised, chilled in ice-cold isotonic (0.25 M) sucrose and weighed. The tissue was disintegrated (2 min, 1500 rev/min, 0°C) in

9 volumes of sucrose (0.25 M) using a Potter-Elvehjem-type homogeniser having an all round clearance between the Perspex pestle and tube of 0.15 mm.

To each of 24 centrifuge tubes, chilled in an ice-bath, were added 0.3 ml of magnesium chloride (0.02 M), 0.3 ml of potassium chloride (1.0 M), 0.2 ml of glycyl glycine buffer (0.3 M, pH 7.4) (McIlwain & Rodnight, 1962c), 0.3 ml of drug or control solution and 0.3 ml of homogenate (10 % W/V in sucrose 0.35 M). Five minutes after the addition of the enzyme source, each tube was transferred to a water bath (25°C) for temperature equilibration (10 min). The order in which tubes were put in the bath for incubation was randomised to avoid biasing the results by variations in technique throughout the experiment. The reaction was started by the addition to each tube of 0.3 ml of adenosinetriphosphate (0.02 M). During incubation (10 min, 25°C) the contents of each tube were exposed to the atmosphere but were not shaken. The reaction was stopped by the addition to each tube of 1.25 ml of perchloric acid (1.0 M). After incubation the tubes were transferred immediately to an ice-bath. Blank

determinations, omitting adenosinetriphosphate, were carried out to estimate the inorganic phosphate present in the homogenate. The mean value obtained for the blanks was subtracted from each of the values obtained for drug and control tubes.

The contents of each tube were centrifuged (5 min, 600 x g, $0^{o}C$) and the supernatant was decanted into a 25 ml volumetric flask. The sediment was washed twice by resuspension in 2, 7.0 ml volumes of deionised water ($0^{o}C$) and recentrifugation (5 min, 600 x g, $0^{o}C$). Each extract was made up to volume (25 ml) with deionised water to produce a final perchloric acid concentration of 0.05 M (Furchgott & De Gubareff, 1956).

The method used for the estimation of inorganic orthophosphate was that of Furchgott and De Gubareff (1956). The technique is a modification of the methods of Fiske and Subbarow (1925) and Lowry and Lopez (1946). The inorganic orthophosphate was determined in weak acid (pH 2.3) with the aid of Cu^{++} ions which increase the rate of development of the reduced phosphomolybdate blue colour. One ml of copper sulphate (0.02 M) dissolved in perchloric acid (0.05 M) was added to each 25 ml of

extract. Two ml of this solution were then pipetted into each spectrophotometer cell of light path 1 cm and capacity 3.0 ml. Using an all-glass tuberculin syringe fitted with a polythene needle, 0.4 ml of Fiske and Subbarow mixture was added to the 2 ml aliquot. The contents of each cell were then stirred briefly using a fine glass rod. Reading against water, at a wavelength of 660 mμ, measurements of optical density were made at 1.0 minute intervals until the reaction was complete as shown by several consecutive readings being the same (3 to 5 minutes). The inorganic orthophosphate liberated in each tube was determined from a calibration graph relating optical density and concentration of inorganic orthophosphate (Fig. 6, page 59).

continued on next page

A number of experiments were carried out using rat brain cerebral cortex slices (100 mg) instead of homogenate. In all other respects these experiments were similar to those in which homogenate was used as a source of enzymic activity.

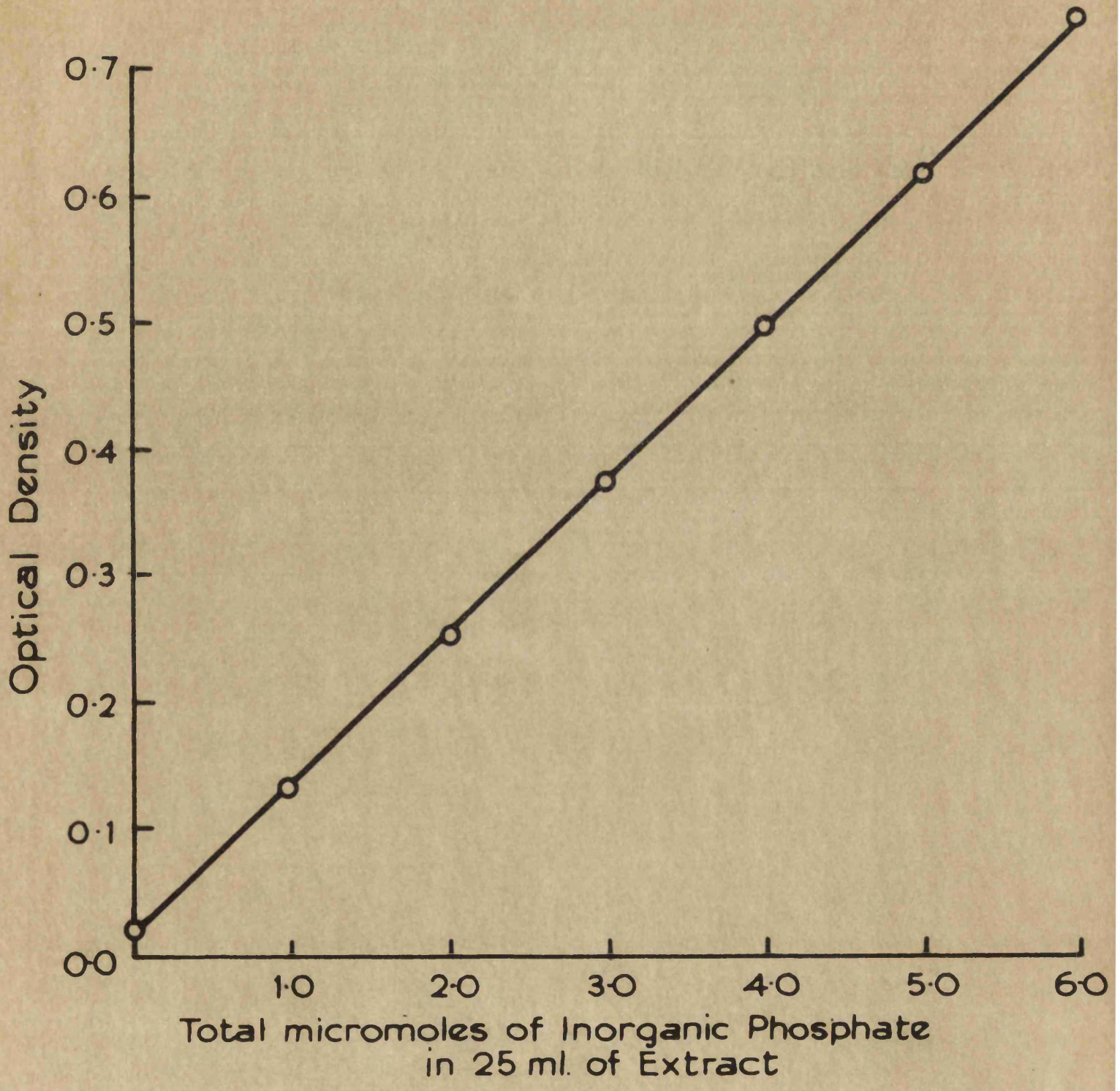

Fig. VI.

Calibration graph for Inorganic Phosphate.
Each Point Represents the Mean of Four Determinations.
A Linear Regression Line was calculated
(see Appendix).

RESULTS

RESULTS

The results are presented under the following headings:

1. Effects upon the oxygen uptake of rat brain cerebral cortex slices, *in vitro*.
2. Effects upon the 2,4-dinitrophenol-stimulated oxygen uptake of rat brain cerebral cortex slices, *in vitro*.
3. Effects upon the monoamine oxidase activity of rat brain mitochondria, *in vitro*.
4. Effects upon the acetylcholinesterase activity of a preparation of rat brain, *in vitro*.
5. Effects upon the adenosinetriphosphatase activity of rat brain cerebral cortex slices and whole brain homogenates, *in vitro*.

Effects Upon the Oxygen Uptake of Rat Brain Cerebral Cortex Slices, *In Vitro*.

The results of the *in vitro* respiration experiments are shown in Table IV (pages 103-107).

d-Amphetamine (5×10^{-5}M) (Fig. 7, page 66) had no effect upon the oxygen uptake of rat brain cerebral cortex slices, even in the fifth hour of the experiment ($0.8 > P > 0.7$). However, *d*-amphetamine (5×10^{-4}M) (Fig. 7, page 66) depressed respiration slightly in the second hour ($0.1 > P > 0.05$) and very significantly ($P < 0.001$) in the succeeding three hours. *l*-Amphetamine, although producing similar effects, was slightly less potent than *d*-amphetamine. Thus, this compound (5×10^{-4}M) inhibited respiration slightly in the third ($0.1 > P > 0.05$) and fourth ($0.1 > P > 0.05$) hours and significantly inhibited respiration in the fifth hour ($0.01 > P > 0.001$) of the experiment. *l*-Amphetamine (5×10^{-3}M) inhibited the oxygen uptake of cerebral cortex slices very significantly ($P < 0.001$) throughout the experiment. On the other hand, methylamphetamine (5×10^{-4}M) had only very slight inhibitory ($0.02 > P > 0.01$)

effects on respiration, even in the third hour after tipping the drug. Furthermore, this drug had no effect, whatsoever, when used at the lower concentration of 5×10^{-5}M). The chemically related compound, ephedrine, had no significant effect on <u>in vitro</u> oxygen uptake at concentrations of 10^{-5}M ($0.5 > P > 0.4$) and 10^{-3}M ($0.4 > P > 0.3$). Similarly, phenmetrazine (Fig. 8, page67), at a concentration of 10^{-3}M, had no significant ($0.3 > P > 0.2$) effect on respiration, even in the fifth hour. However, the other phenylethylamine derivative, WIN 19,583-4, although inactive at the lower concentration (0.8×10^{-5}M), inhibited the oxygen uptake significantly ($0.01 > P > 0.001$) in the fifth hour, when used at the higher concentration of 0.8×10^{-3}M.

Iproniazid (10^{-5}M) had no effect on respiration. At the higher dose level of 10^{-3}M, however, it significantly ($0.05 > P > 0.02$) inhibited the oxygen uptake in the fourth hour. The chemically closely related compound, isoniazid, had no significant effect on respiration at concentrations of 10^{-5}M and 10^{-3}M. These results may be contrasted with the effects

obtained with pheniprazine, a drug which is chemically related both to the phenylethylamine derivatives, amphetamine and ephedrine and also to the hydrazine derivative, iproniazid. This compound (10^{-3}M) depressed the respiration of rat brain cerebral cortex slices very significantly ($P < 0.001$), even in the second hour of the experiment. However, at the lower dose level of 10^{-5}M, pheniprazine had no significant ($0.1 > P > 0.05$) effect on respiration in the fourth hour of the experiment.

The iminodibenzyl derivative, imipramine (4×10^{-3}M) (Fig. 9, page 68), depressed respiration very significantly ($P < 0.001$) throughout the period of its contact with the tissue. Imipramine (4×10^{-4}M) (Fig. 9, page 68) very significantly ($P < 0.001$) increased the oxygen uptake in the second hour of the experiment, an effect which was subsequently reversed in the third hour, when the respiration was significantly ($0.02 > P > 0.01$) depressed. This latter effect became more pronounced in the last two hours of the experiment ($P < 0.001$). The chemically related compound amitriptyline (4×10^{-3}M) also inhibited the oxygen

uptake very significantly ($P < 0.001$), even in the first hour after tipping. In contrast to the stimulant effect on the oxygen uptake, produced in the second hour of the experiment by the lower dose of imipramine, amitriptyline, when used at the same concentration (4×10^{-4}M), had only a very slight effect, which was not significant ($0.6 > P > 0.5$). It did, however, inhibit the oxygen uptake very significantly ($P < 0.001$) during the last three hours of the experiment.

Sodium barbitone (10^{-3}M) (Fig. 10, page 69) had no significant effect on the respiration of rat brain cerebral cortex slices. It did, however, depress the oxygen uptake at the very high concentration of 10^{-2}M. At this dose level, sodium barbitone inhibited respiration very significantly ($P < 0.001$) in the third hour and throughout the remaining two hours of the experiment.

The respiration of cerebral cortex slices was significantly ($P < 0.001$) stimulated throughout the experiment by the uncoupling agent, 2,4-dinitrophenol (2.5×10^{-5}M).

The centrally-acting drugs studied, inhibited the respiration of rat brain cerebral cortex slices only at concentrations which were very much higher than those likely to be found in the brain following the administration of therapeutic doses of these drugs. While, however, most of the central nervous system stimulants inhibited the oxygen uptake, at least at concentrations of 10^{-3}M (e.g. WIN 19,583-4, 0.8 x 10^{-3}M; iproniazid, 10^{-3}M), and others inhibited respiration at much lower dose levels (e.g. pheniprazine, 10^{-5}M; d-amphetamine, 5 x 10^{-4}M; amitriptyline, 4 x 10^{-4}M), the weak central stimulants, isoniazid, ephedrine and phenmetrazine had no effect even at very high concentrations. An exception was l-amphetamine, which was almost equipotent in inhibiting respiration with d-amphetamine. Sodium barbitone inhibited the oxygen uptake only at the extremely high concentration of 10^{-2}M.

Among the compounds investigated, only the uncoupling agent, 2,4-dinitrophenol and imipramine, caused an increase in the rate of respiration. The increased oxygen uptake caused by imipramine was observed only in the second hour of the experiment.

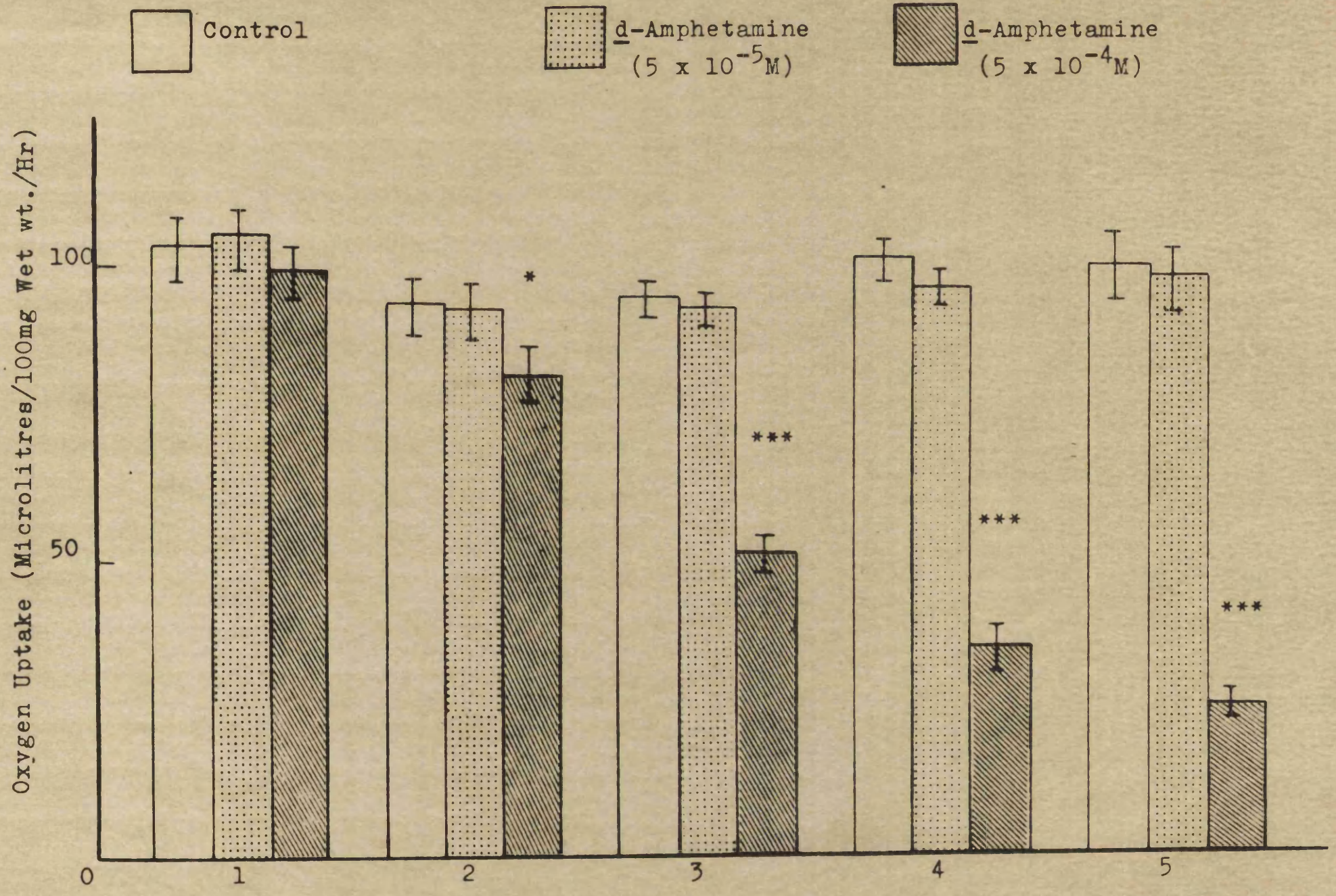
Control
d-Amphetamine (5 x 10⁻⁵M)
d-Amphetamine (5 x 10⁻⁴M)
Oxygen Uptake (Microlitres/100mg Wet wt./Hr)
100
50
0
1
2
3
4
5
*
***
***
***
Hour of Experiment

Fig. VII

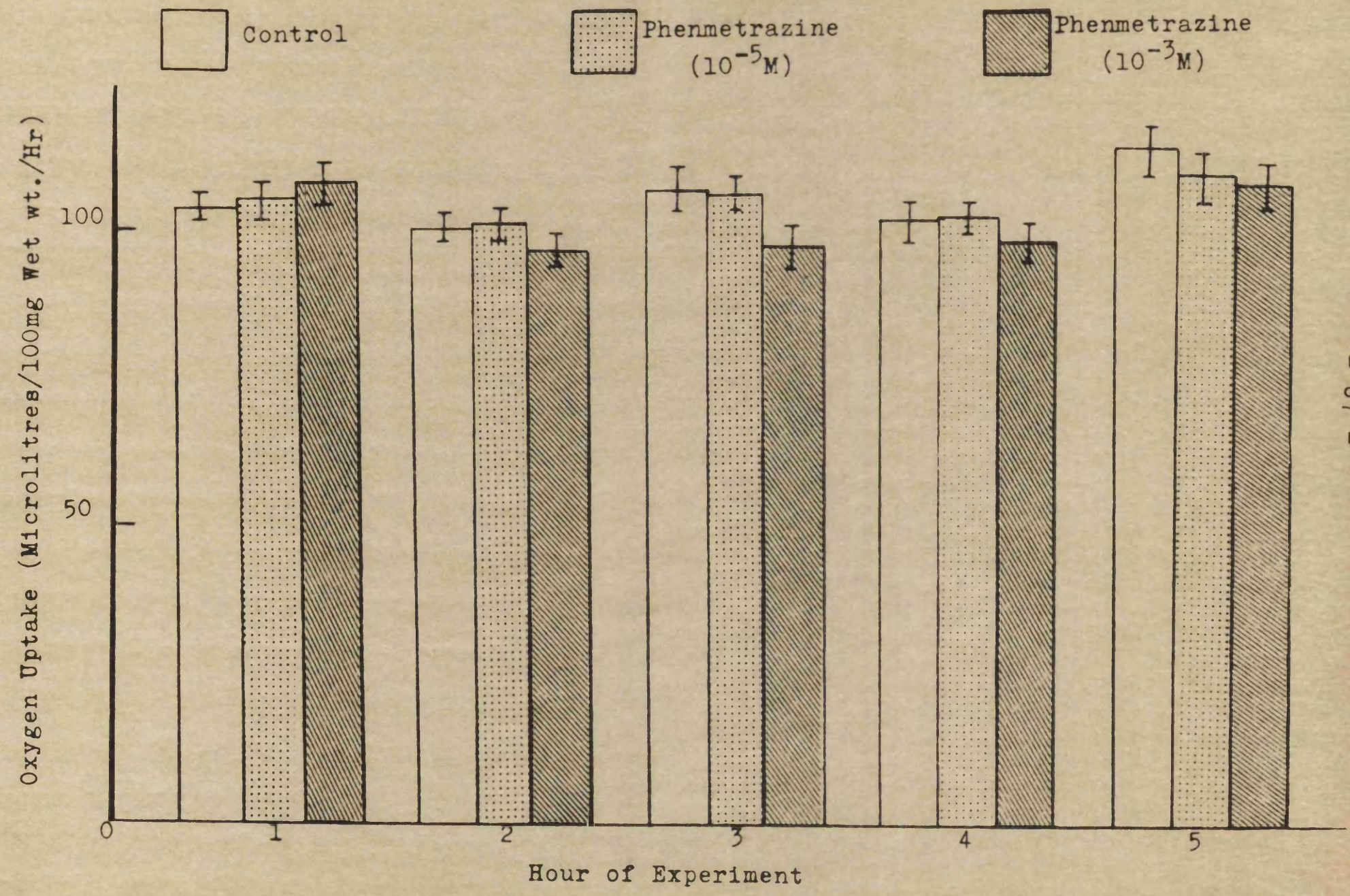
Control
Phenmetrazine (10^{-5}M)
Phenmetrazine (10^{-3}M)
Oxygen Uptake (Microlitres/100mg Wet wt./Hr)
100
50
0
1
2
3
4
5
Hour of Experiment

Fig. VIII

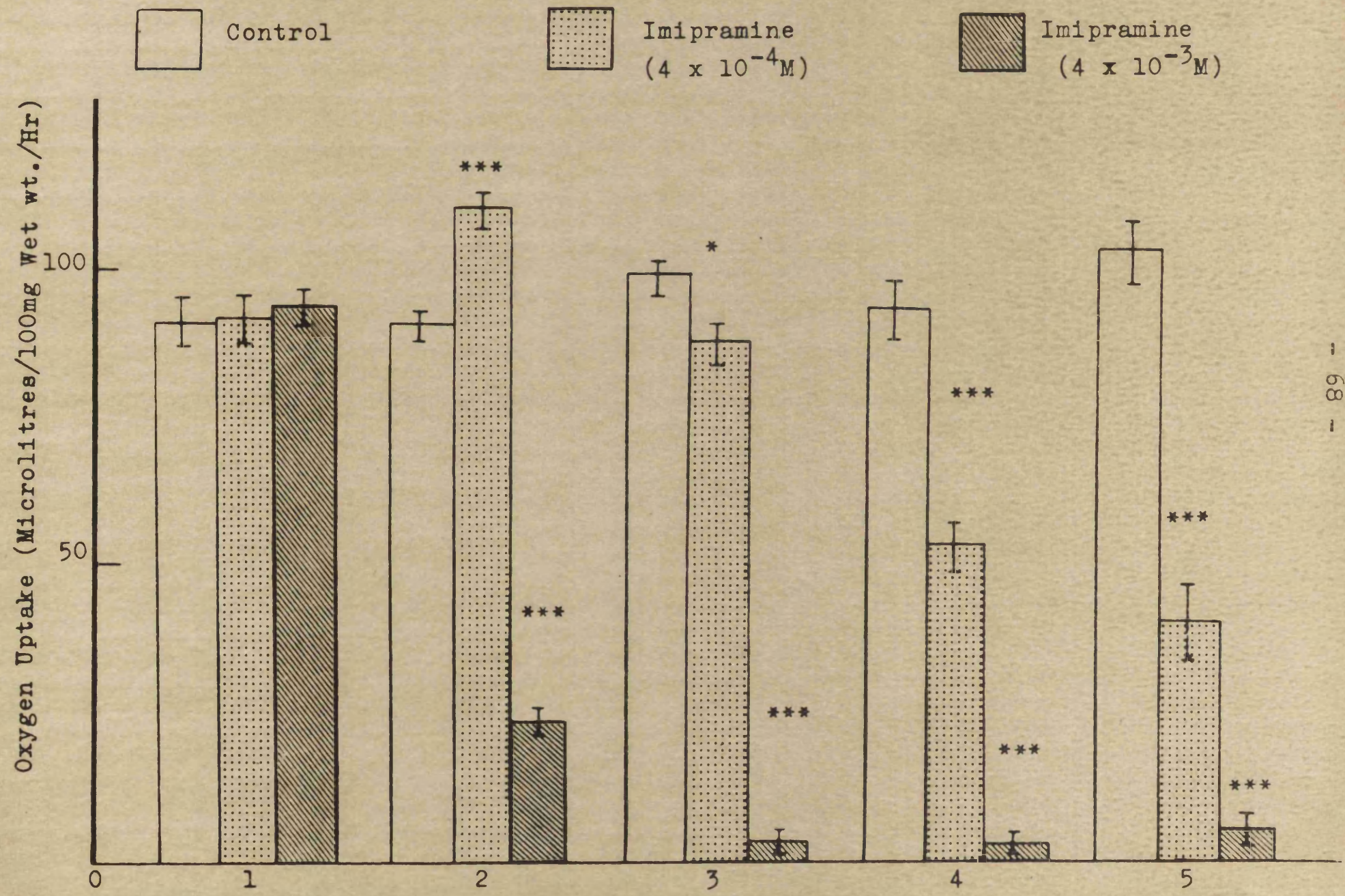
Control
Imipramine
(4 x 10^{-4}M)
Imipramine
(4 x 10^{-3}M)
Oxygen Uptake (Microlitres/100mg Wet wt./Hr)
100
50
0
1
2
3
4
5
Hour of Experiment
***
*
***
***
***
***
***

Fig. IX

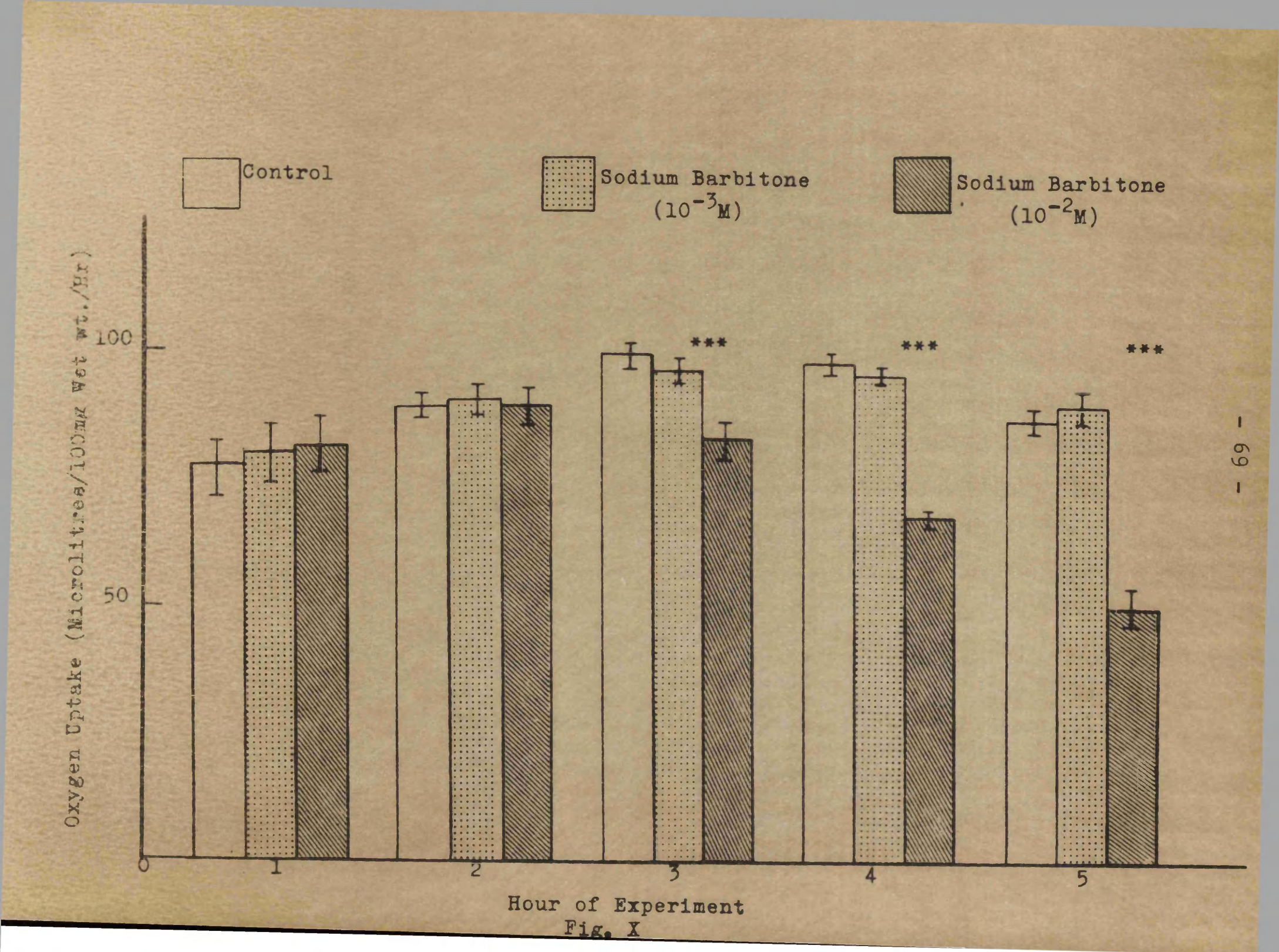
Control
Sodium Barbitone (10^{-3}M)
Sodium Barbitone (10^{-2}M)
Oxygen Uptake (Microlitres/100mg Wet wt./Hr)
100
50
0
1
2
3
4
5
***
***
***
Hour of Experiment

Fig. X

Effects Upon the 2,4-Dinitrophenol Stimulated Oxygen Uptake of Rat Brain Cerebral Cortex Slices, In Vitro.

The results of the in vitro 2,4-dinitrophenol-stimulated respiration experiments are shown in Table V (page 108).

Ephedrine (10^{-3}M) had no effect upon the 2,4-dinitrophenol-stimulated oxygen uptake of rat brain cerebral cortex slices, even in the third hour of the experiment ($0.2 > P > 0.1$). The chemically related compound, phenmetrazine (10^{-3}M), was also without effect even after four hours ($0.6 > P > 0.5$). WIN 19,583-4 had no effect, when used at a concentration of 10^{-4}M ($0.9 > P > 0.8$). However, at the higher dose level of 10^{-3}M, WIN 19,583-4 depressed the oxygen uptake very significantly in the third and fourth hours of the experiment ($P < 0.001$). This effect was very much more marked than that observed after five hours, in experiments using unstimulated respiration ($0.01 > P > 0.001$).

Pheniprazine (10^{-4}M) inhibited the oxygen uptake in the second ($0.02 > P > 0.01$), third ($P < 0.001$)

and fourth ($P < 0.001$) hours of the experiment. Isoniazid (10^{-3}M) markedly depressed the oxygen uptake in the third ($0.01 > P > 0.001$) and fourth hours ($P < 0.001$) of the experiment. This effect may be contrasted with the inactivity of isoniazid, in experiments using unstimulated respiration (Table IV, page 105).

Amitriptyline (4×10^{-4}M) inhibited the 2,4-dinitrophenol-stimulated respiration of rat brain cerebral cortex slices in the first hour ($P < 0.001$) and in the succeeding three hours of the experiment. On the other hand, sodium barbitone (5×10^{-4}M) had no significant effect on the oxygen uptake in this investigation.

In the experiments on stimulated respiration, a number of drugs which had previously had an inhibitory effect on normal <u>in vitro</u> oxygen uptake, produced a more marked response (e.g. pheniprazine). While some drugs which had no significant effect (e.g. isoniazid) or just barely significant effects (e.g. WIN 19,583-4) on normal unstimulated respiration

in vitro, significantly inhibited stimulated respiration, other compounds (e.g. ephedrine and phenmetrazine) had no effect. These observations, however, confirm the view that stimulated *in vitro* respiration is more susceptible to inhibition than normal unstimulated oxygen uptake.

Effects Upon the Monoamine Oxidase Activity of Rat Brain Mitochondria, In Vitro.

A comparison of the in vitro monoamine oxidase inhibitory activities of the central nervous system stimulant drugs and chemically related compounds investigated, is given in Table I (page 75) . These values were calculated from graphs (pages 76-81) relating the percentage inhibition of the enzyme to the molar concentration of the drug used. The potency of each compound is expressed as the negative logarithm of the molar concentration producing 50% inhibition of the enzyme (the PI_{50} value).

In this investigation, the least active compound was ephedrine (Fig. 14, page 77), which had a PI_{50} value of 2.18. The other phenylethylamine derivatives, phenmetrazine (Fig. 19, page 80), WIN 19,583-4 (Fig. 21, page 81), l-amphetamine (Fig. 13, page 77), methylamphetamine (Fig. 18, page 79) and d-amphetamine (Fig. 12, page 76), together with isoniazid (Fig. 17, page 79) were also inactive. These drugs had PI_{50} values ranging from 2.53 to 3.16.

The iminodibenzyl derivatives, imipramine (Fig 15, page 78) and amitriptyline (Fig. 11, page 76) were moderately potent inhibitors of this enzyme, having respectively PI_{50} values of 3.94 and 3.83. The most active inhibitors of rat brain mitochondrial monoamine oxidase activity were iproniazid (Fig. 16, page 78) (PI_{50} = 5.83) and tranylcypromine (Fig. 20, page 80) (PI_{50} = 6.34).

It is apparent from these results that while all the compounds having potent monoamine oxidase inhibitory activity are in fact central nervous system stimulants, not all of the central stimulant drugs studied, are potent inhibitors of monoamine oxidase. Thus, the central stimulant compounds, d-amphetamine and WIN 19,583-4, were almost equiactive with l-amphetamine, which has very little central nervous system stimulant activity. It is also interesting to note that the antidepressives, imipramine and amitriptyline, are more active inhibitors of monoamine oxidase than the amphetamines.

Table I.

Drug	PI_{50} Value
Ephedrine	2.18
Isoniazid	2.53
Phenmetrazine	2.72
WIN 19,583-4	2.83
l-Amphetamine	3.00
Methylamphetamine	3.00
d-Amphetamine	3.16
Amitriptyline	3.83
Imipramine	3.94
Iproniazid	5.83
Tranylcypromine	6.34

A comparison of the PI_{50} Values for Monoamine Oxidase, Obtained Using Rat Brain Mitochondria.

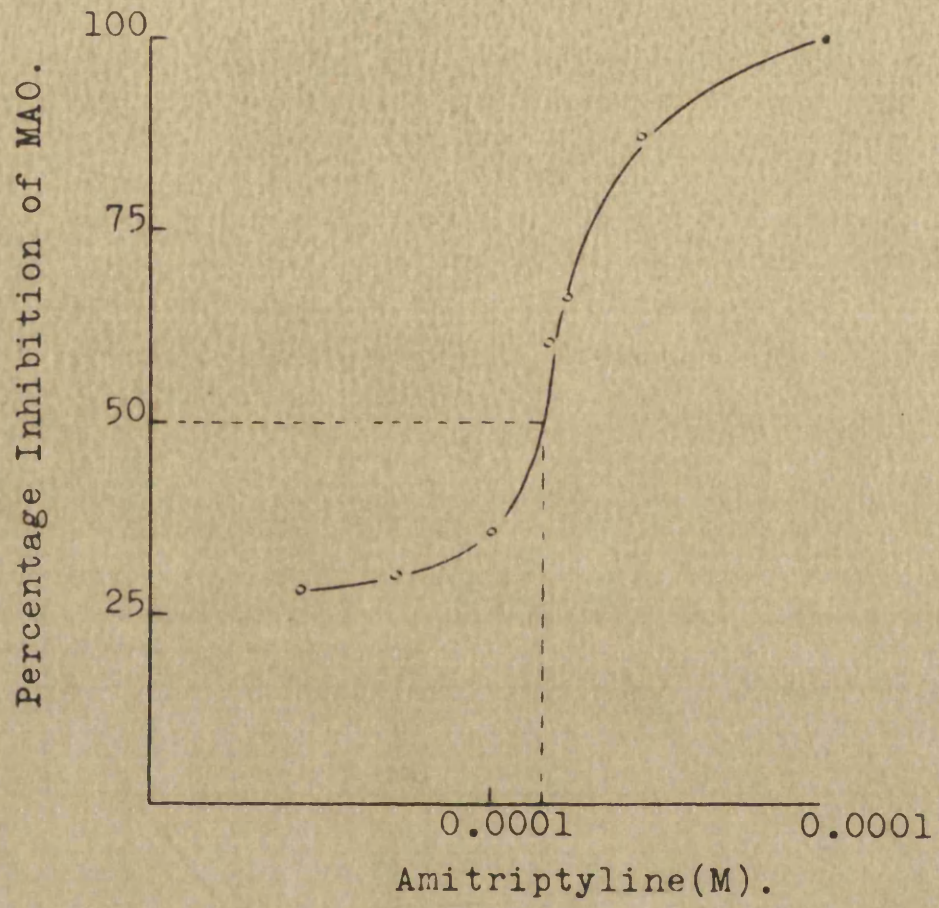

Fig. XI

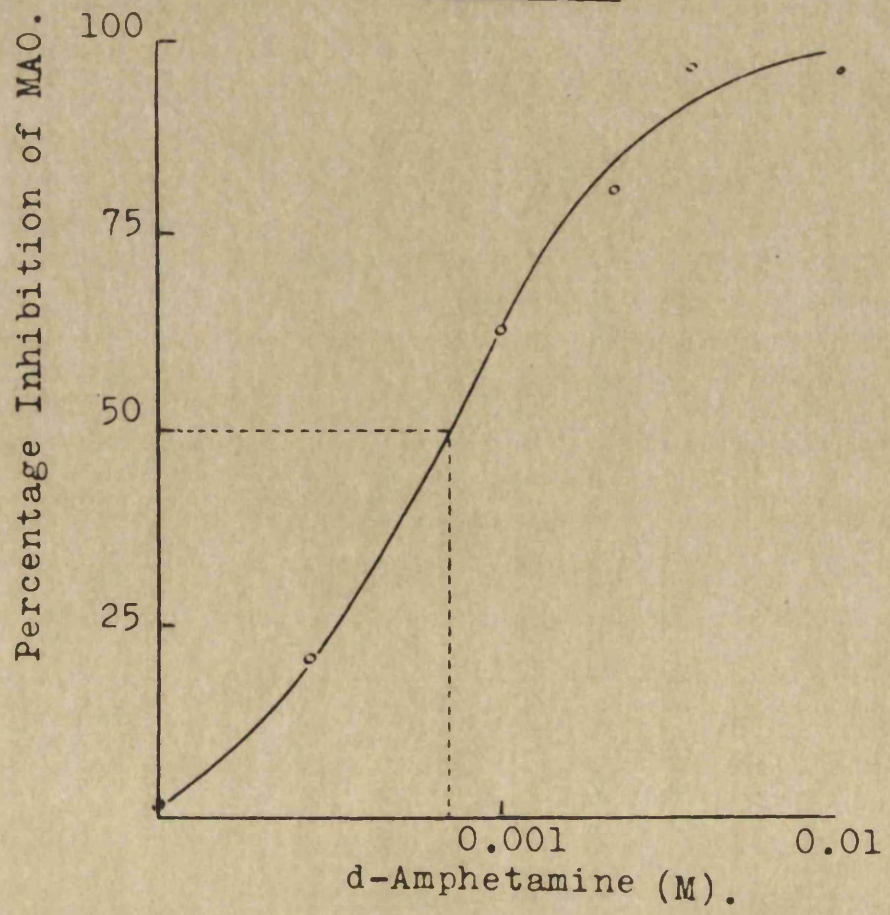

Fig. XII

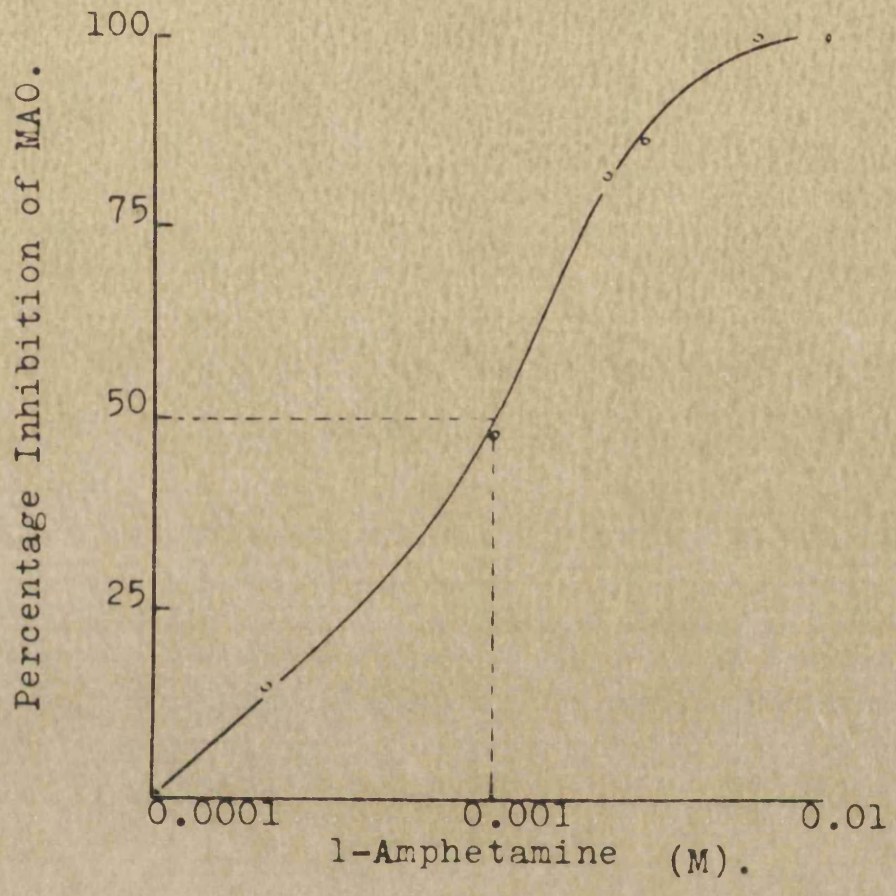

Fig. XIII

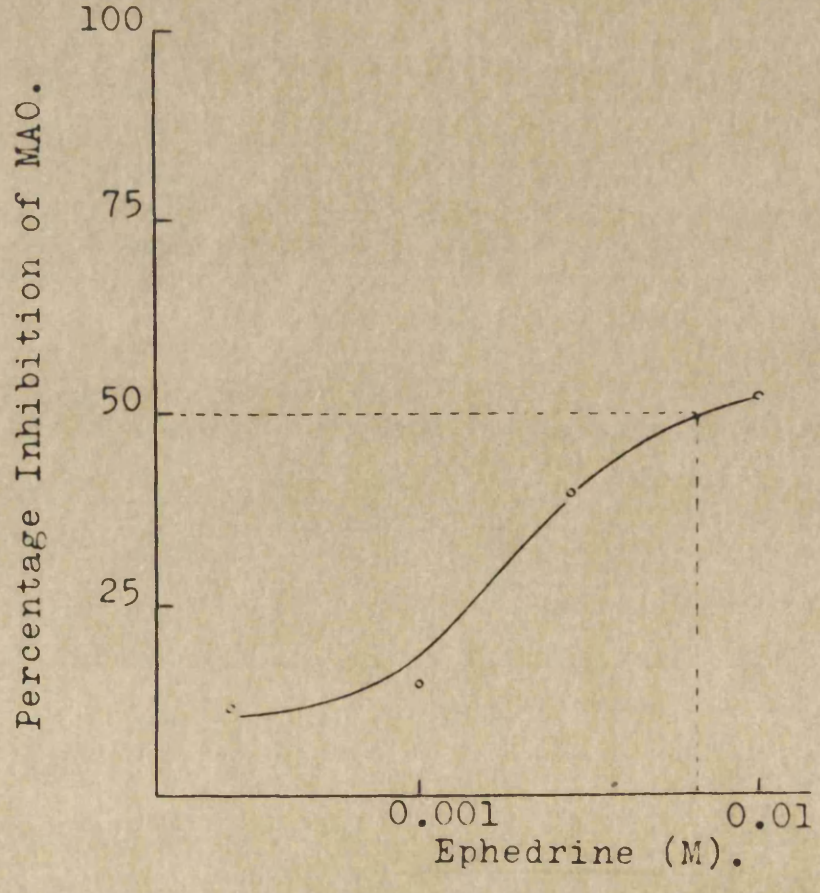

Fig. XIV

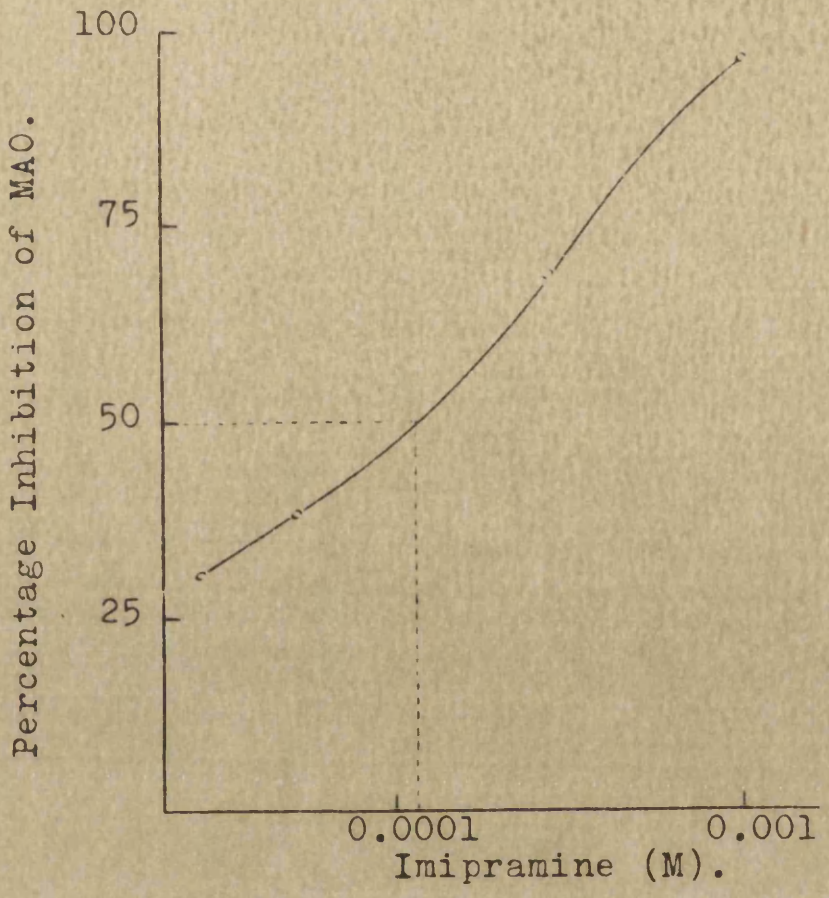

Fig. XV

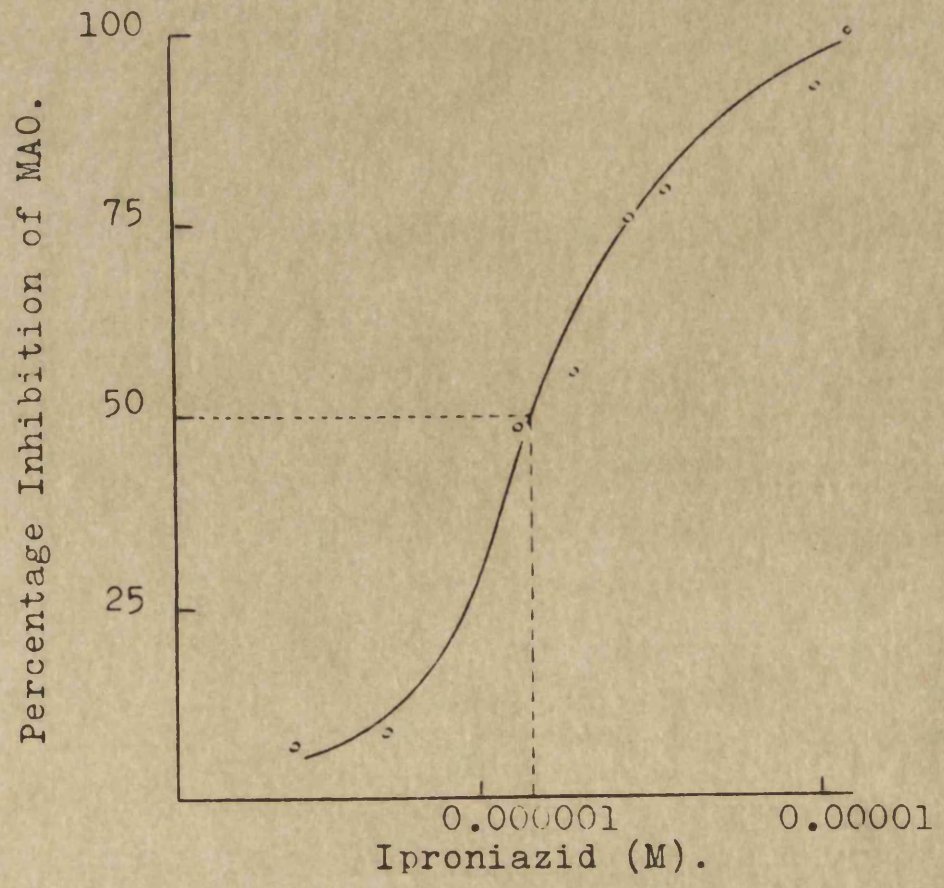

Fig. XVI

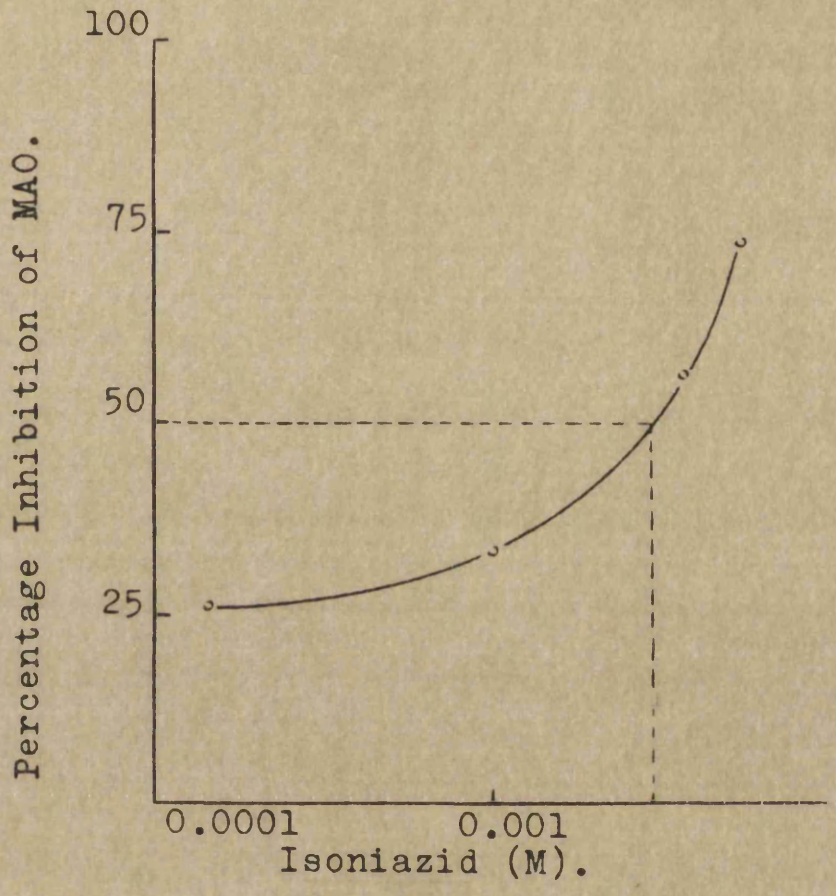

Fig. XVII

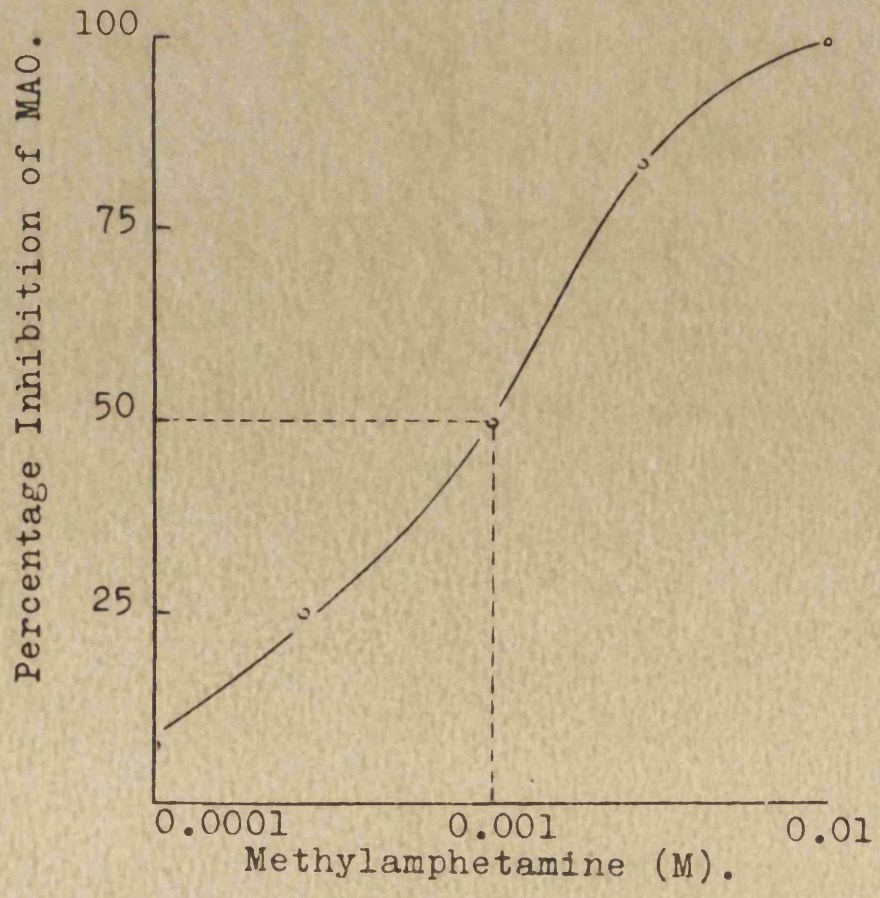

Fig. XVIII

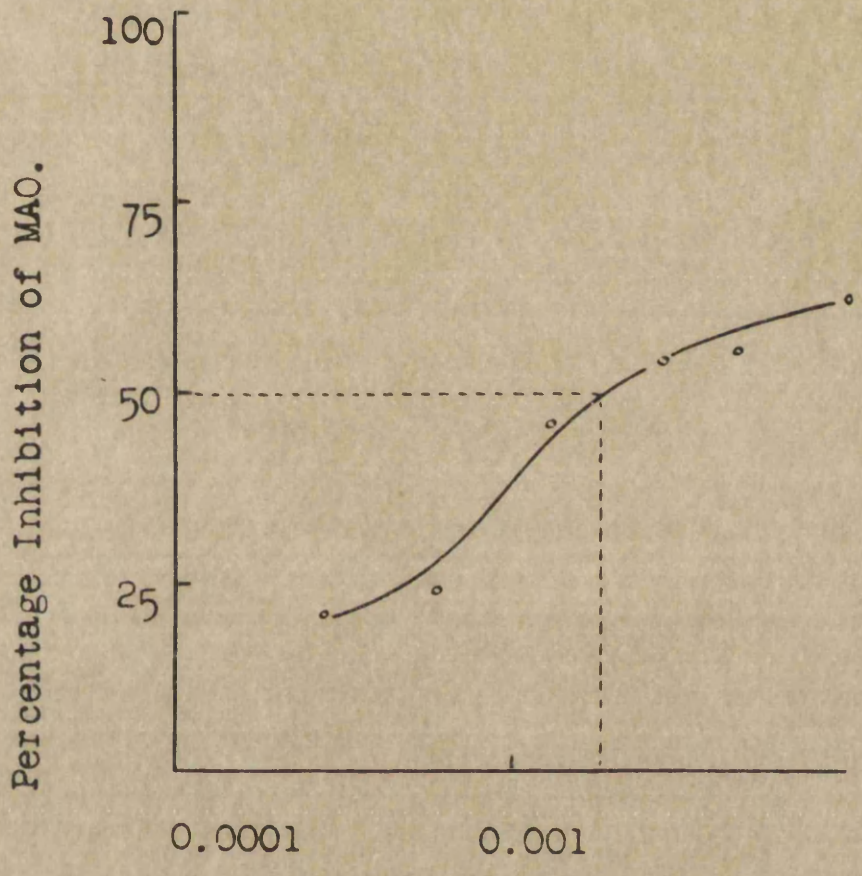

Fig. XIX

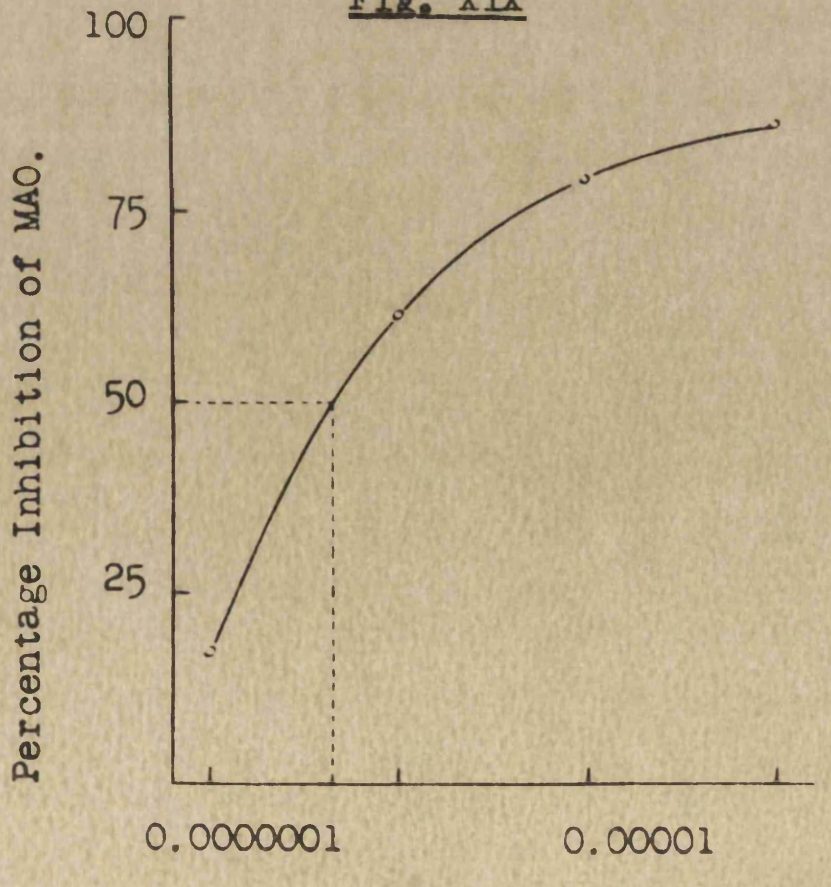

Fig. XX

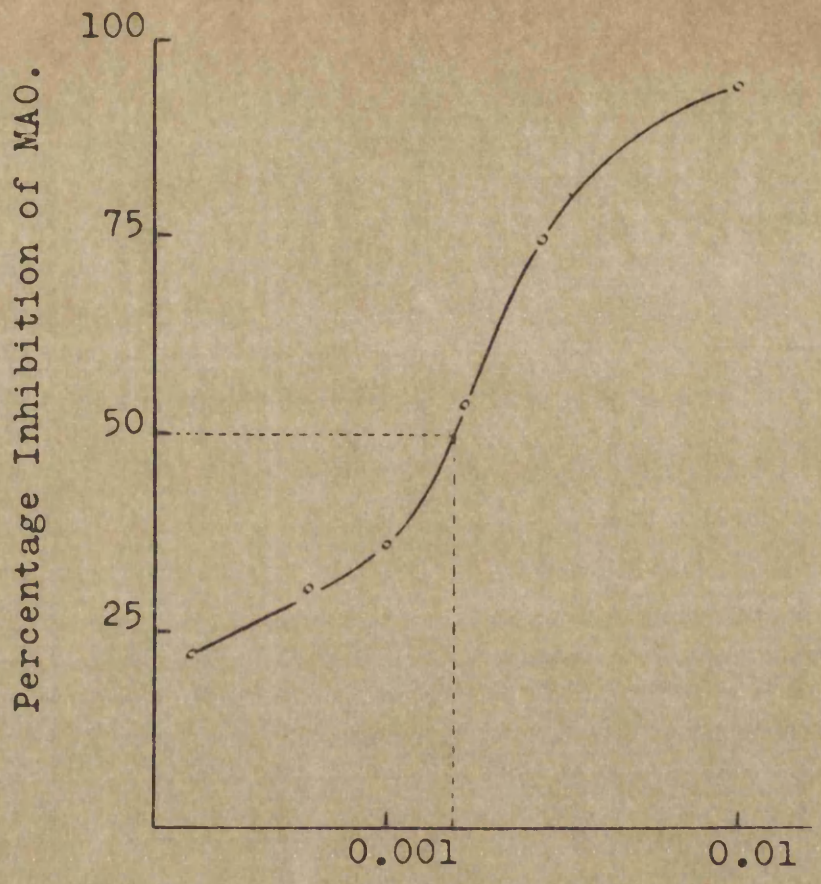
Percentage Inhibition of MAO.
100
75
50
25
0.001
0.01
Win 19,583 (M).

Fig. XXI

Effects Upon the Acetylcholinesterase Activity of a Preparation of Rat Brain, In Vitro.

A comparison of the in vitro anti-acetylcholinesterase activities of the centrally-acting drugs studied, is given in Table II(page 84) The PI_{50} values were calculated from graphs (pages 85 - 90) relating the percentage inhibition of the enzyme to the molar concentration of the drug used.

None of the compounds investigated had marked in vitro acetylcholinesterase inhibitory activity. The least active drugs were l-amphetamine (Fig.24, page 86), d-amphetamine (Fig. 22, page 85), WIN 19,583-4 (Fig. 32, page 90) and isoniazid (Fig. 28, page 88), all of which had PI_{50} values not greater than 2.0. Ephedrine (Fig. 26, page 87), phenmetrazine (Fig. 30, page 89), iproniazid (Fig. 29, page 88), pheniprazine (Fig. 25, page 86) and the central nervous system depressant, sodium barbitone (Fig. 31, page 89) had PI_{50} values ranging from 2.0 to 2.77. The most potent in vitro inhibitors of rat brain acetylcholinesterase activity were the iminodibenzyl derivatives, imipramine (Fig. 27,

page 87) amd amitriptyline (Fig. 23, page 85), which had PI_{50} values of 3.0 and 3.03 respectively.

The anti-acetylcholinesterase activities of the drugs investigated were in general extremely low. The potent central nervous system stimulants, d-amphetamine, WIN 19,583-4 and methyl amphetamine had very similar PI_{50} values to those of the weaker central stimulants, l-amphetamine and isoniazid. Furthermore, the potent antidepressives, iproniazid and pheniprazine inhibited *in vitro* brain acetylcholinesterase activity to approximately the same extent as the central nervous system depressant, sodium barbitone.

Table II.

Drug	PI_{50} Value
Isoniazid	2.0
l-Amphetamine	2.0
d-Amphetamine	2.0
WIN 19,583-4	2.0
Pheniprazine	2.46
Iproniazid	2.50
Sodium Barbitone	2.55
Phenmetrazine	2.72
Ephedrine	2.77
Imipramine	3.00
Amitriptyline	3.03

A comparison of the PI_{50} Values for Acetylcholinesterase, Obtained Using A Preparation of Rat Whole Brain Homogenate.

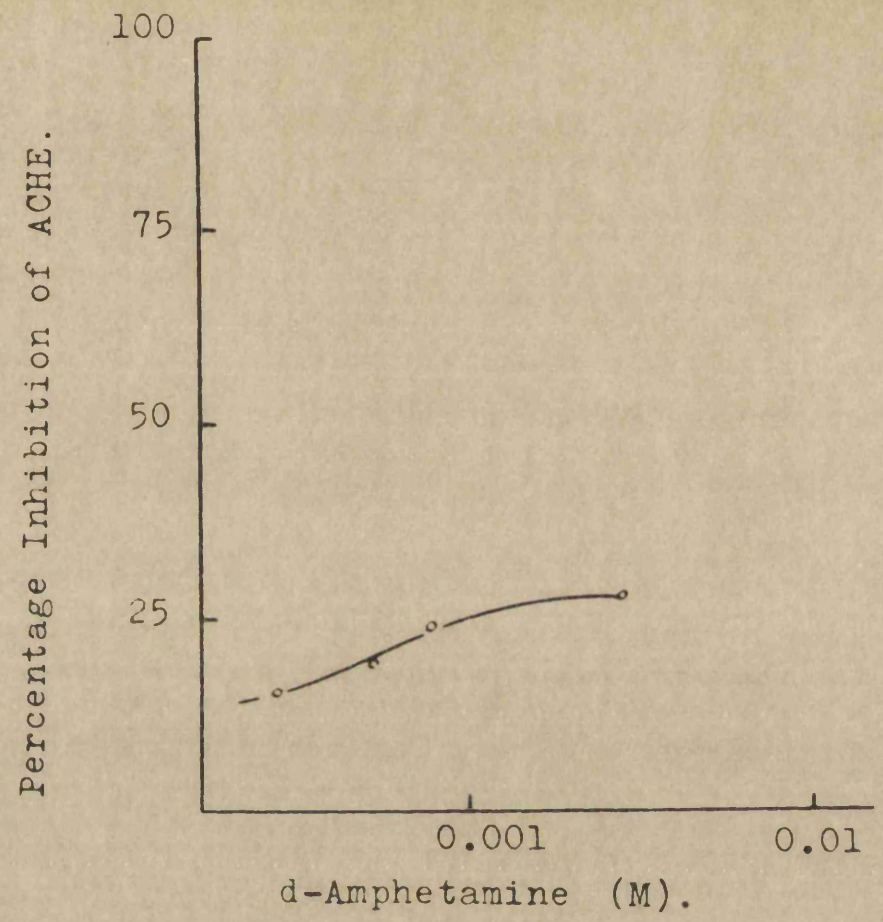

Fig. XXII

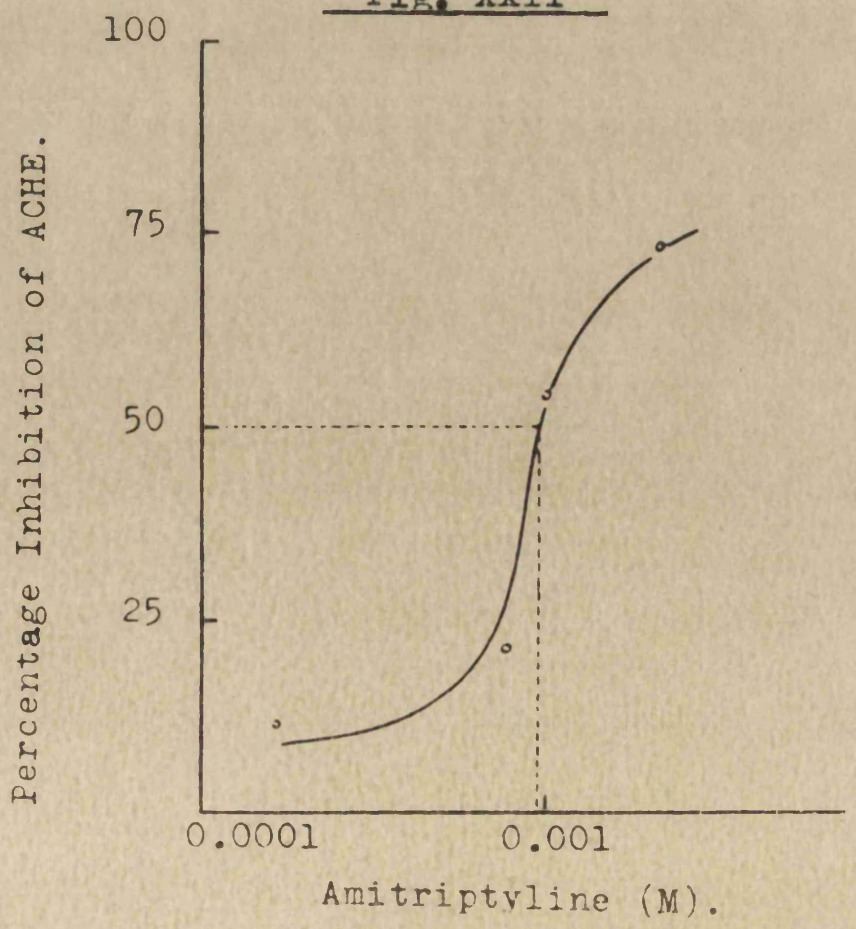

Fig. XXIII

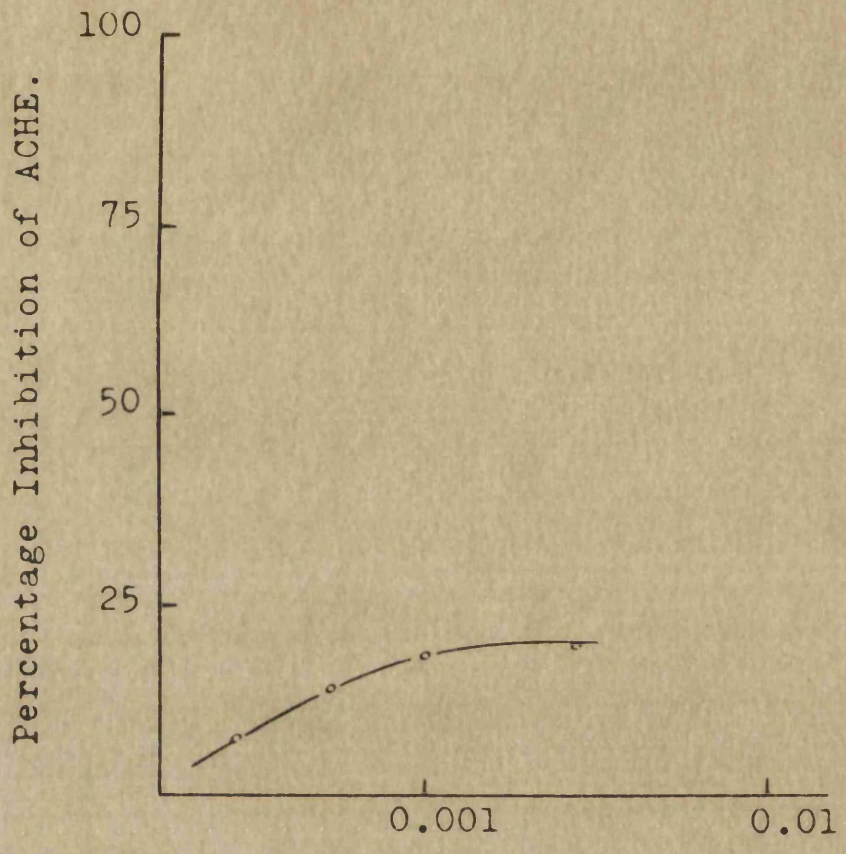

Fig. XXIV

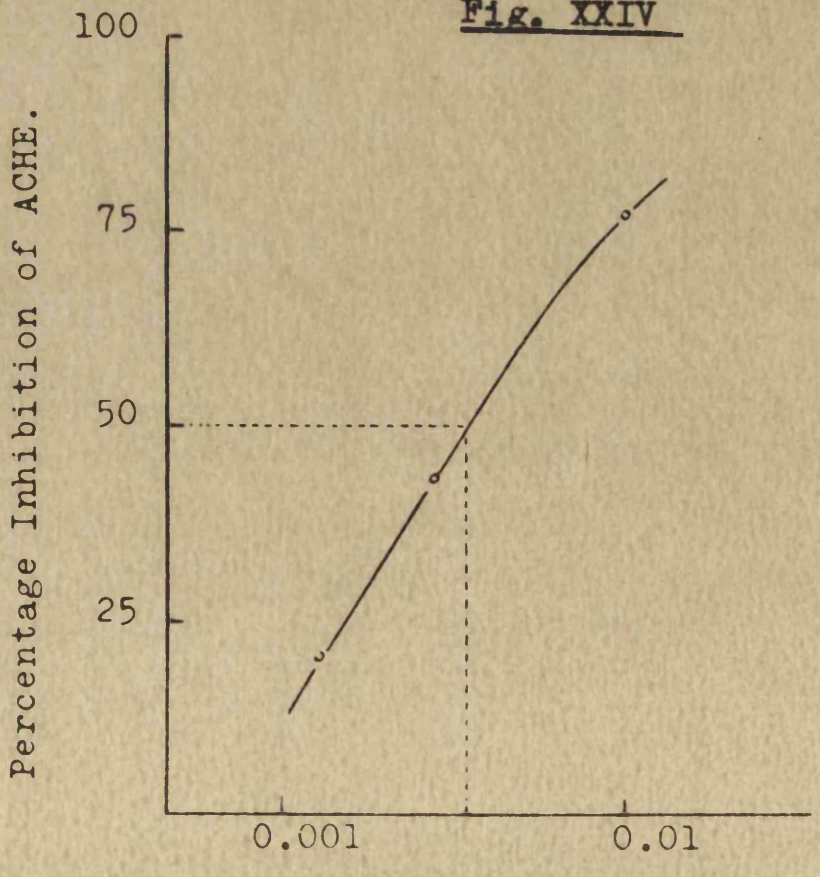

Fig. XXV

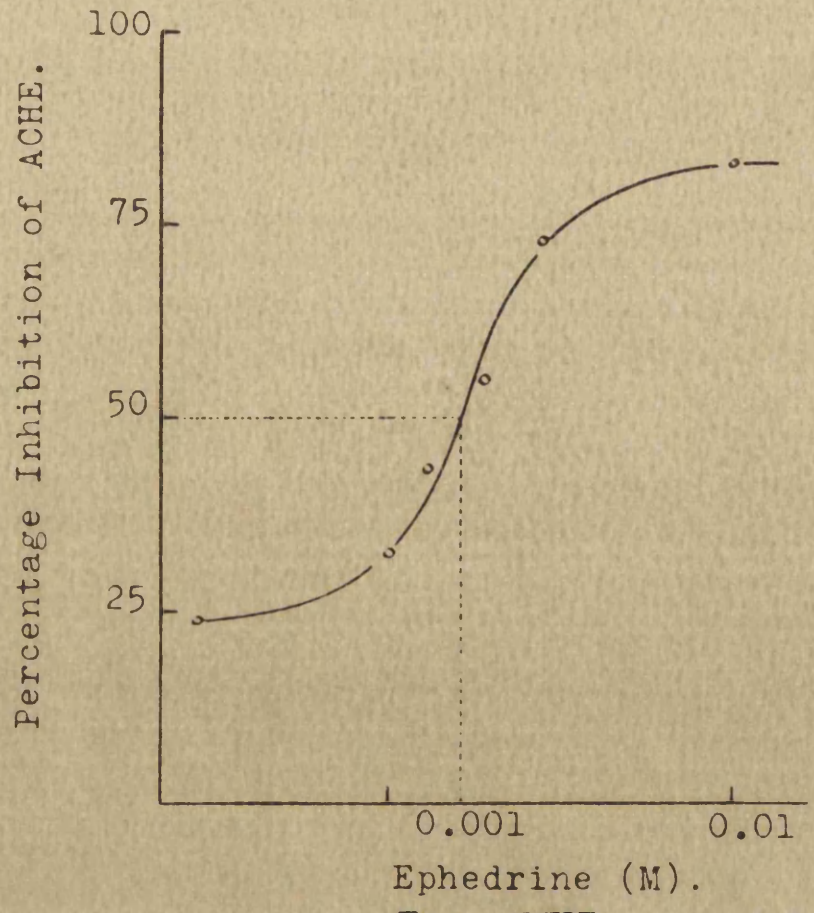

Fig. XXVI

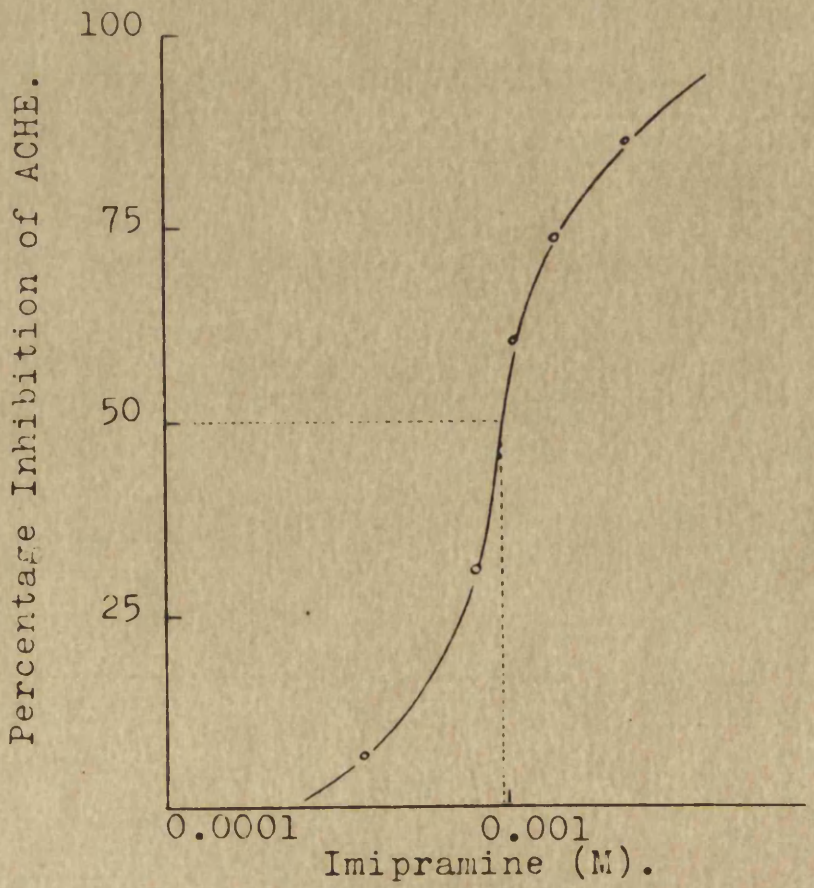

Fig. XXVII

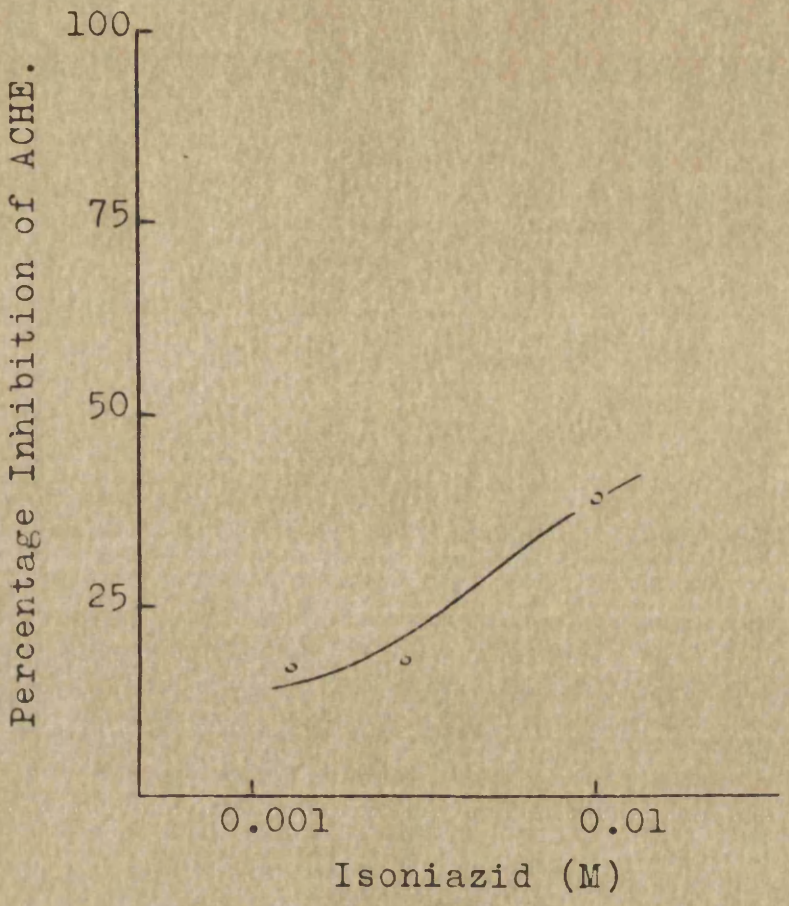

Fig. XXVIII

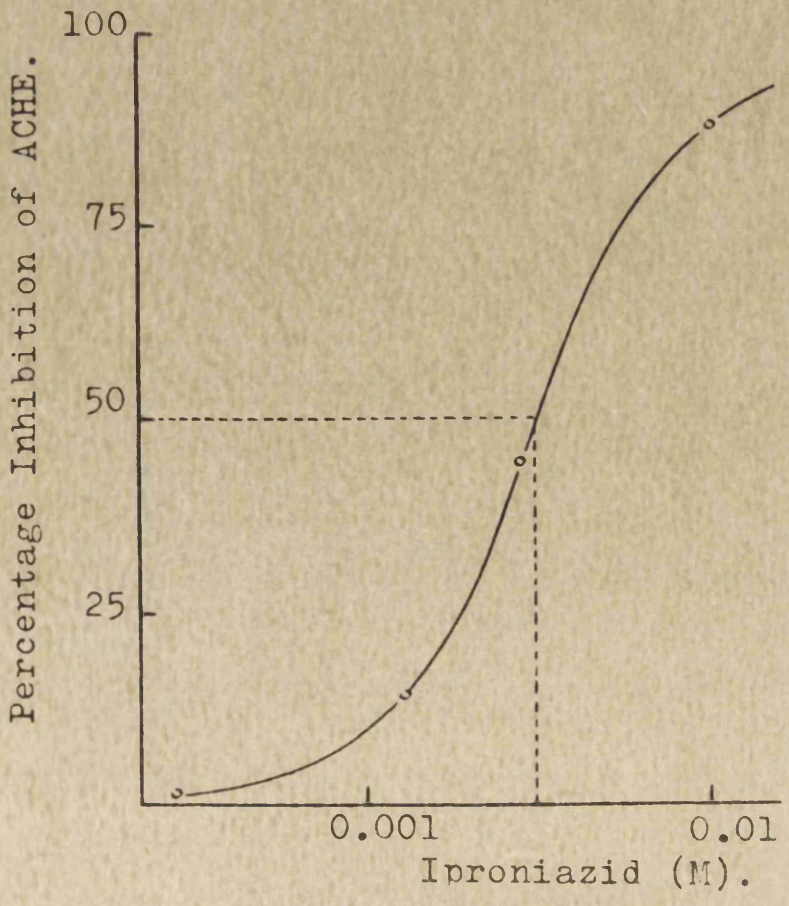

Fig. XXIX

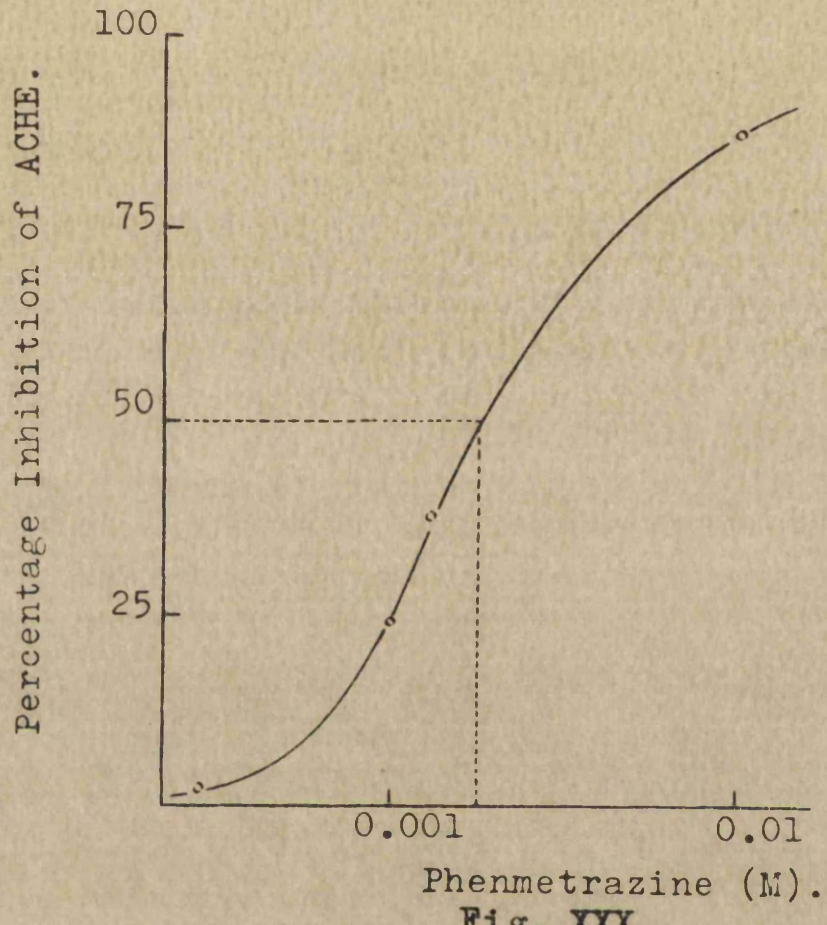

Fig. XXX

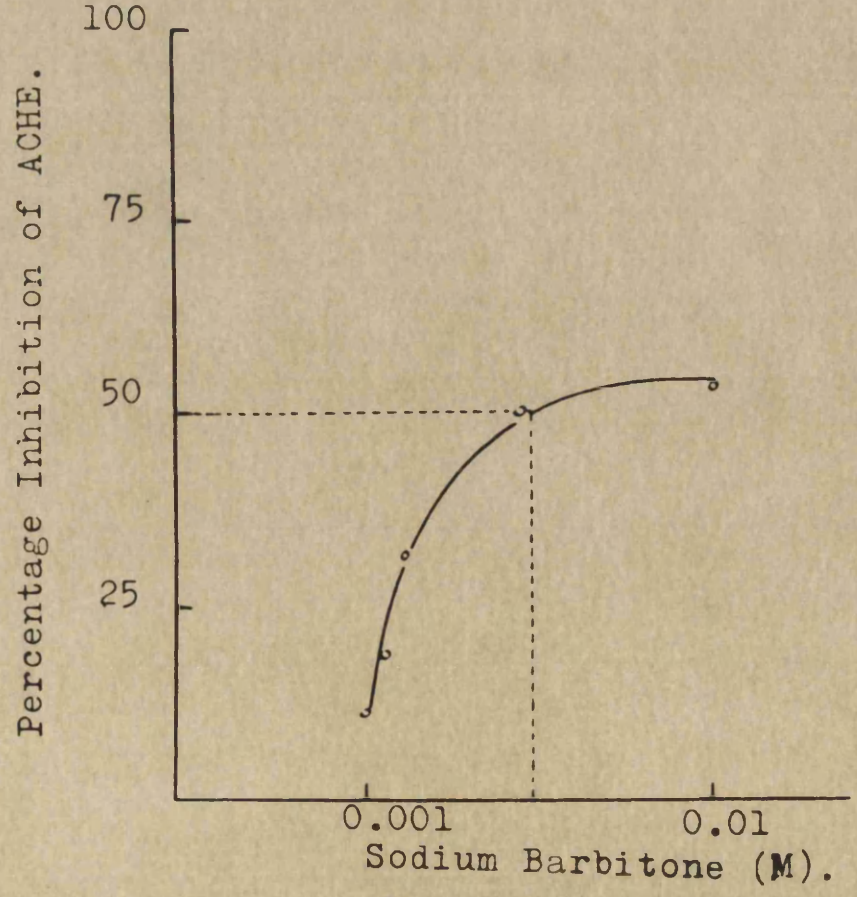

Fig. XXXI

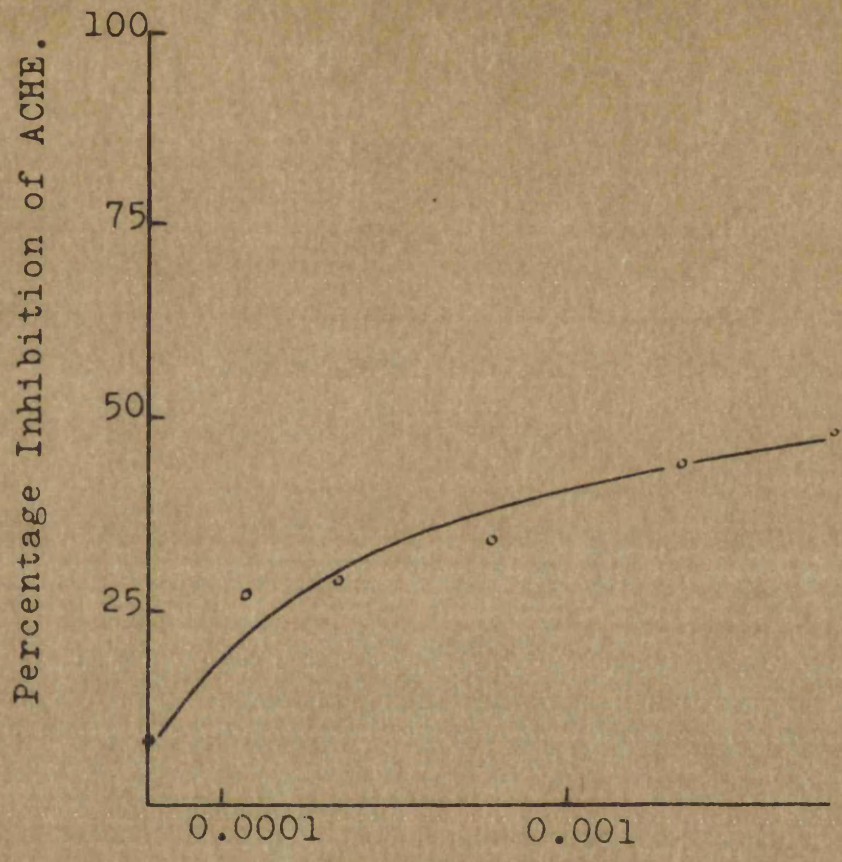
Percentage Inhibition of ACHE.
100
75
50
25
0.0001
0.001
Win 19,583 (M).

Fig. XXXII

Effects Upon the Adenosinetriphosphatase Activity of Rat Brain Cerebral Cortex Slices and Whole Brain Homogenates, In Vitro.

A comparison is shown in Table III(page 93) of the effects of a number of central nervous system stimulant drugs, some chemically related compounds and the classic uncoupling agent, 2,4-dinitrophenol, on the amount of inorganic phosphate liberated by rat whole brain homogenates and cerebral cortex slices in vitro.

The adenosinetriphosphatase activity of cerebral cortex slices was stimulated by 2,4-dinitrophenol (3×10^{-4} M) (Fig. 33, page 94). This effect is shown in Table III by the significant ($0.05 > P > 0.02$) increase in the amount of inorganic phosphate liberated in the presence of this compound. Ephedrine, however, even at the high concentration of 10^{-2} M had no effect ($0.8 > P > 0.7$) on the in vitro enzymic hydrolysis of adenosinetriphosphate (Fig. 34, page 95). On the other hand, d-amphetamine (10^{-2} M) (Fig. 35, page 96) inhibited the adenosinetriphosphatase activity of rat

brain cerebral cortex slices, an effect indicated in Table III(page 93) by the highly significant (P 0.001) fall in the amount of inorganic phosphate liberated.

In the experiments using whole brain homogenates, d-amphetamine (10^{-2} M)(Fig. 37, page 98) significantly ($P < 0.001$) inhibited the adenosine-triphosphatase activity to approximately the same extent (14%) as observed in the experiments using slices of rat brain cerebral cortex. However, phenmetrazine (10^{-2} M) (Fig. 36, page 97), WIN 19,583-4 (10^{-3} M) (Fig. 41, page 102) and tranylcypromine (10^{-3} M) (Fig. 38, page 99) had no significant ($P > 0.9$; $0.5 > P > 0.4$; $0.9 > P > 0.8$ respectively) effects upon the activity of this enzyme in vitro. The drug which had the most pronounced effect on rat brain adenosinetriphosphatase activity was imipramine (Figs. 39, 40) (pages 100,101). This compound markedly inhibited ($P < 0.001$) this enzyme in rat brain homogenates at concentrations of 10^{-3} M (Fig. 39, page 100) and 10^{-4} M (Fig. 40, page 101).

Table III

Drug (M)	Drug Treated	Control	P Value
2,4-Dinitrophenol * 3×10^{-4}	1.946 ± 0.075	1.618 ± 0.119	$0.05 > P > 0.02$
Ephedrine * 10^{-2}	1.482 ± 0.074	1.472 ± 0.068	$0.8 > P > 0.7$
d-Amphetamine * 10^{-2}	1.350 ± 0.0102	1.572 ± 0.0104	$P < 0.001$
Phenmetrazine † 10^{-2}	2.203 ± 0.060	2.209 ± 0.071	$P > 0.9$
Tranylcypromine † 10^{-3}	2.807 ± 0.058	2.820 ±0.037	$0.9 > P > 0.8$
Imipramine † 10^{-3}	2.088 ± 0.088	4.134 ± 0.113	$P < 0.001$
Imipramine † 10^{-4}	3.868 ± 0.039	4.174 ± 0.051	$P < 0.001$
WIN 19,583-4 † 10^{-3}	3.648 ± 0.169	3.496 ± 0.094	$0.5 > P > 0.4$

The Effects of a Number of Centrally-Acting Drugs, some Chemically Related compounds and 2,4-Dinitrophenol upon the Adenosinetriphosphatase Activity of Rat Brain Cerebral Cortex Slices (*) and Whole Brain Homogenates (†), In Vitro.
The Results are in Micromoles of Inorganic Phosphate Liberated ± (S.E. of Mean)/100 mg Wet Wt. of Slices (*) or 0.3 ml Homogenate (10% W/V) (†)/Hour.
The Significance of the differences from the Control Mean in Each Experiment is given by the P Value.

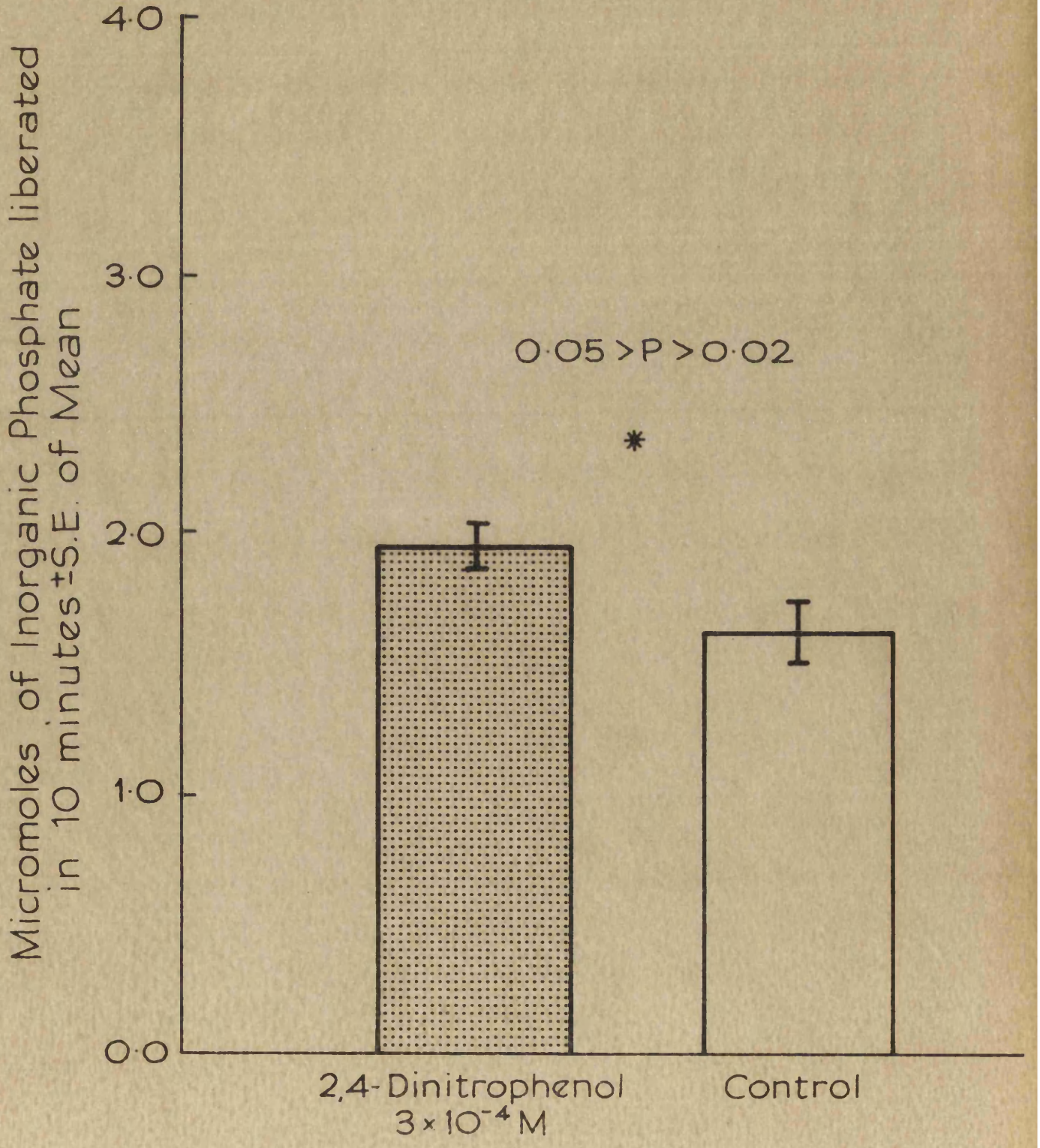
Micromoles of Inorganic Phosphate liberated in 10 minutes ± S.E. of Mean
4·0
3·0
2·0
1·0
0·0
0·05 > P > 0·02
*
2,4-Dinitrophenol
3 × 10⁻⁴ M
Control

Fig. XXXIII

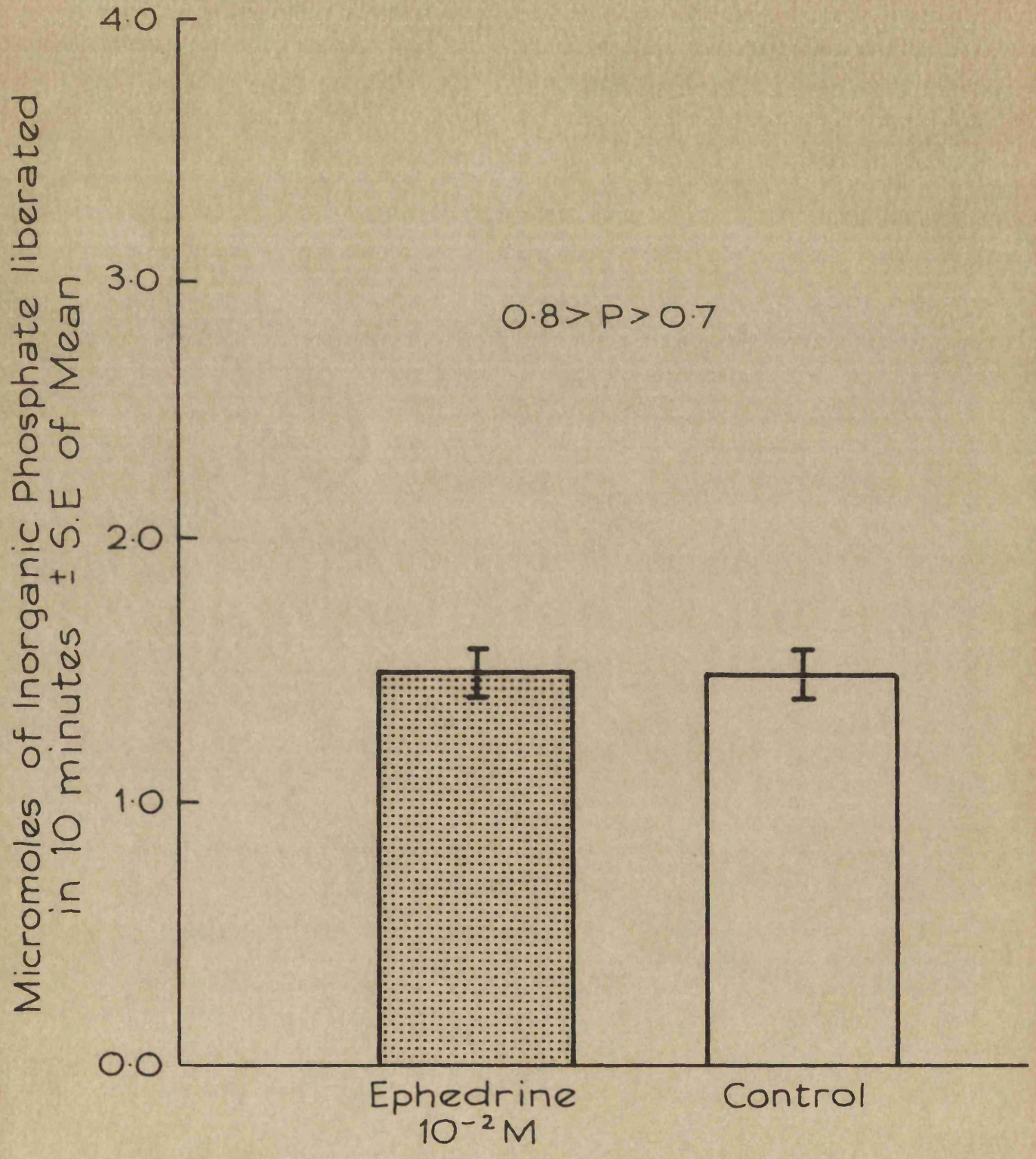
Micromoles of Inorganic Phosphate liberated in 10 minutes ± S.E of Mean
4·0
3·0
2·0
1·0
0·0
0·8 > P > 0·7
Ephedrine 10^{-2}M
Control

Fig. XXXIV

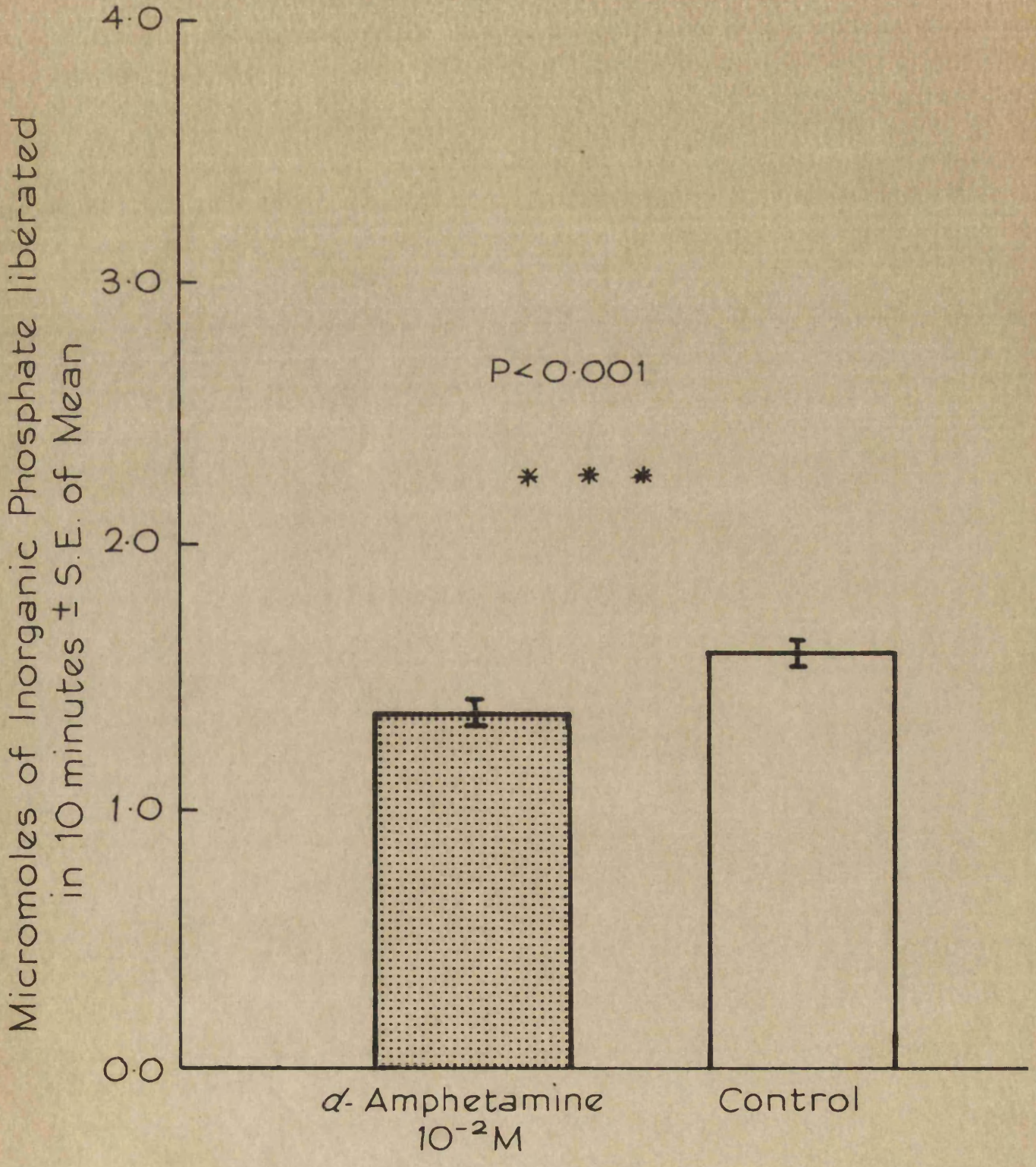
Micromoles of Inorganic Phosphate liberated in 10 minutes ± S.E. of Mean
4·0
3·0
2·0
1·0
0·0
P< 0·001
* * *
α- Amphetamine 10⁻²M
Control

Fig. XXXV

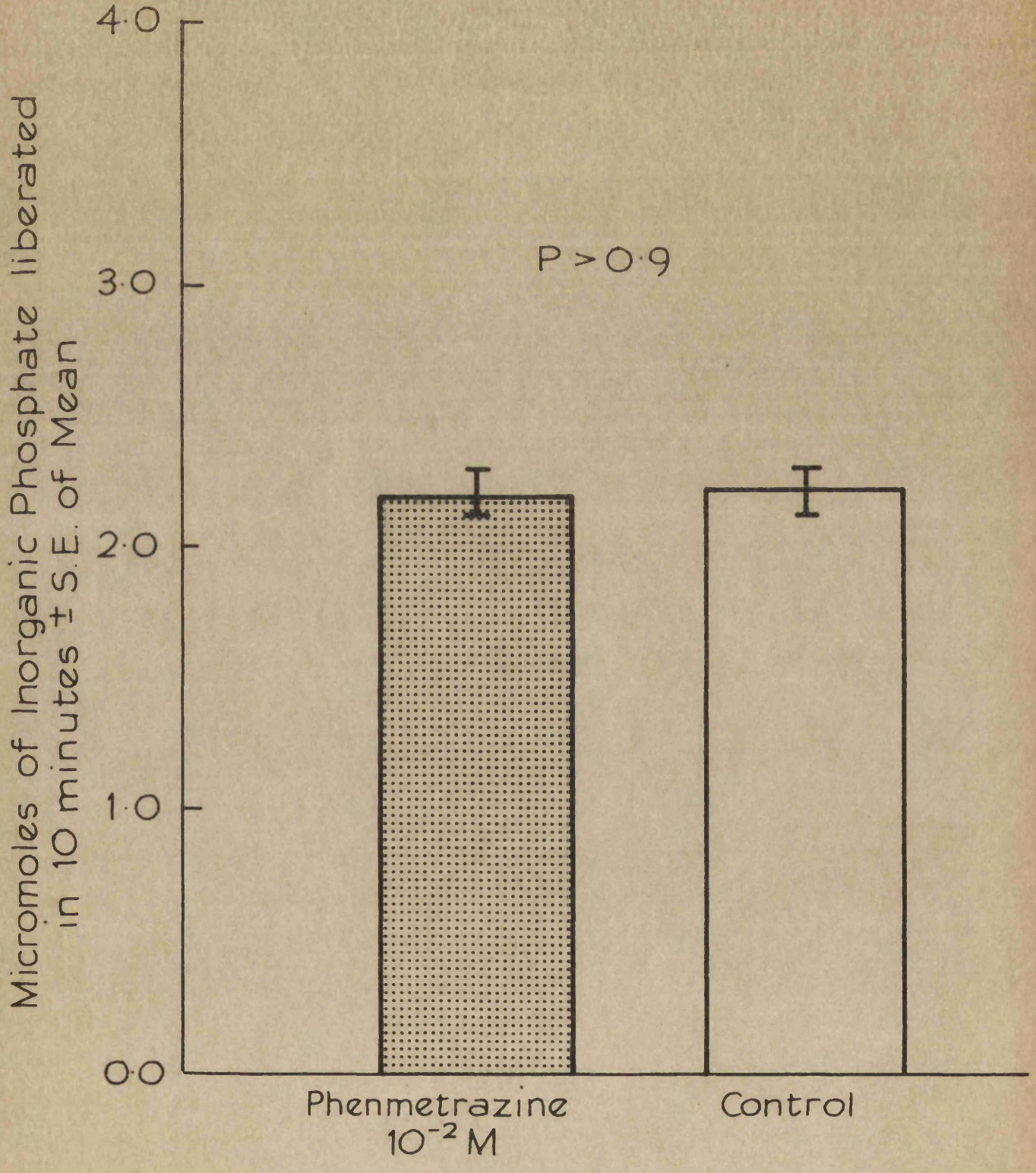
4·0
3·0
2·0
1·0
0·0
Micromoles of Inorganic Phosphate liberated in 10 minutes ± S.E. of Mean
P > 0·9
Phenmetrazine 10^{-2} M
Control

Fig. XXXVI

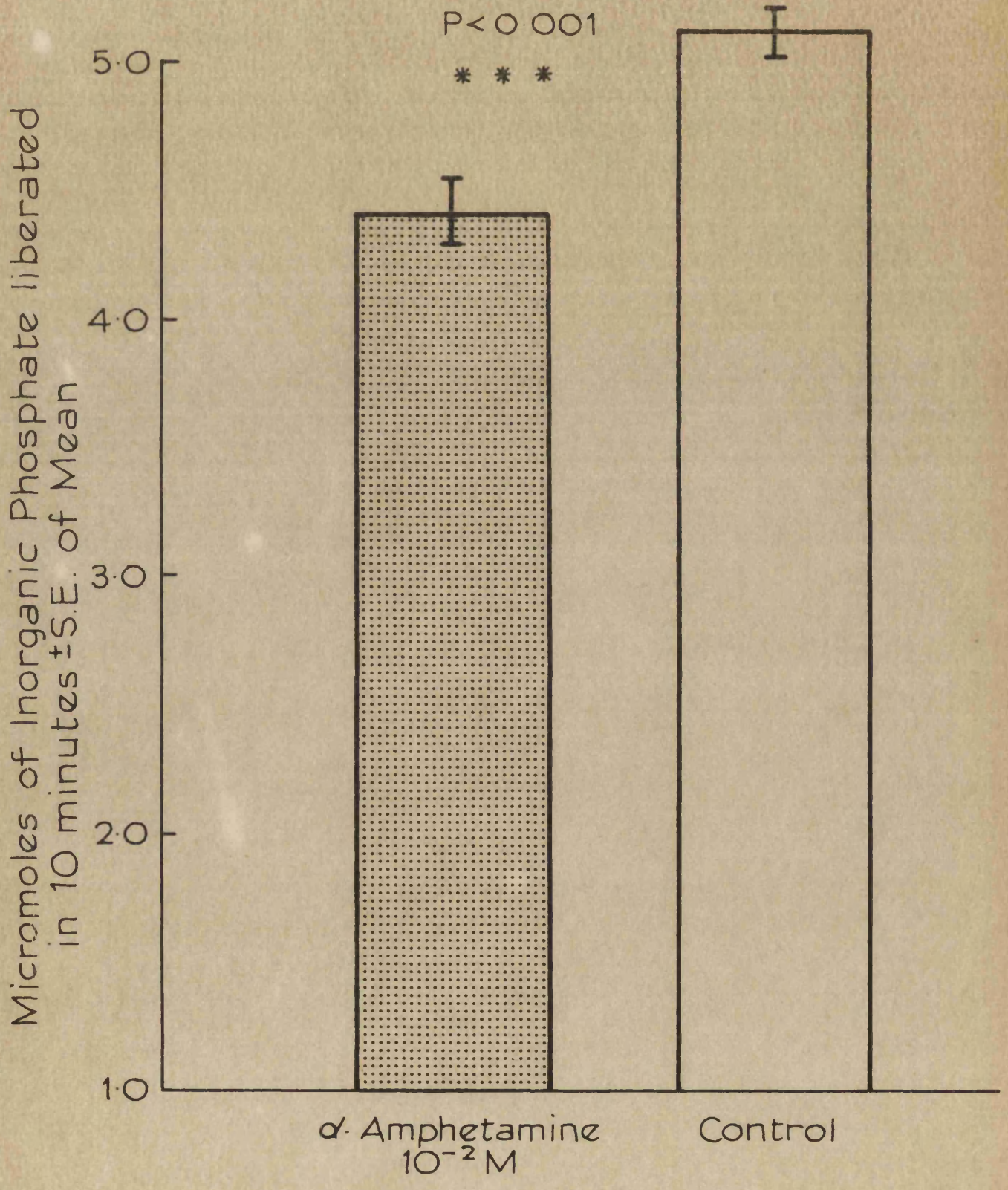
P<0·001
* * *
5·0
4·0
3·0
2·0
1·0
Micromoles of Inorganic Phosphate liberated in 10 minutes ±S.E. of Mean
α. Amphetamine 10^{-2} M
Control

Fig. XXXVII

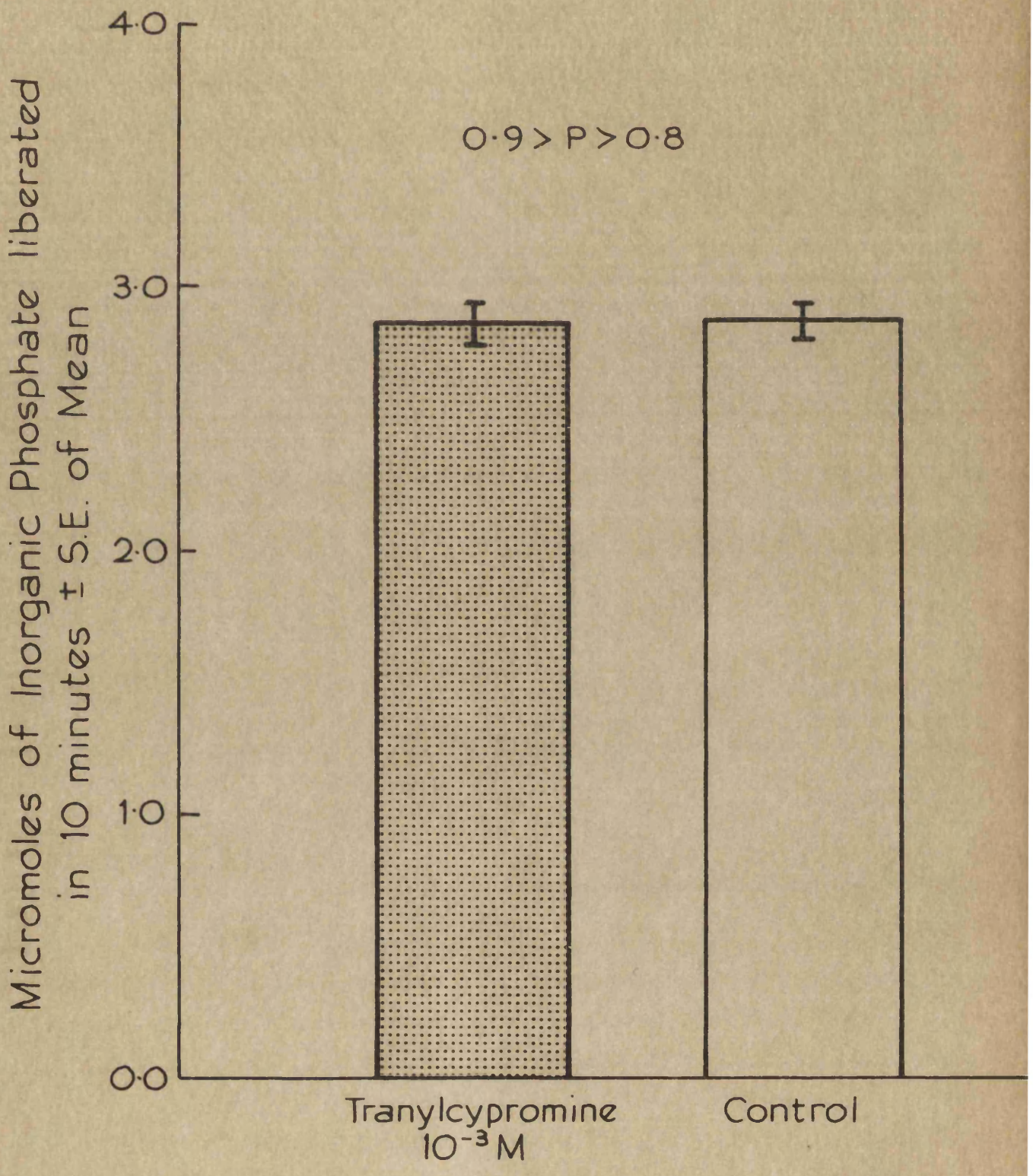
Micromoles of Inorganic Phosphate liberated in 10 minutes ± S.E. of Mean
4·0
3·0
2·0
1·0
0·0
0·9 > P > 0·8
Tranylcypromine 10⁻³ M
Control

Fig. XXXVIII

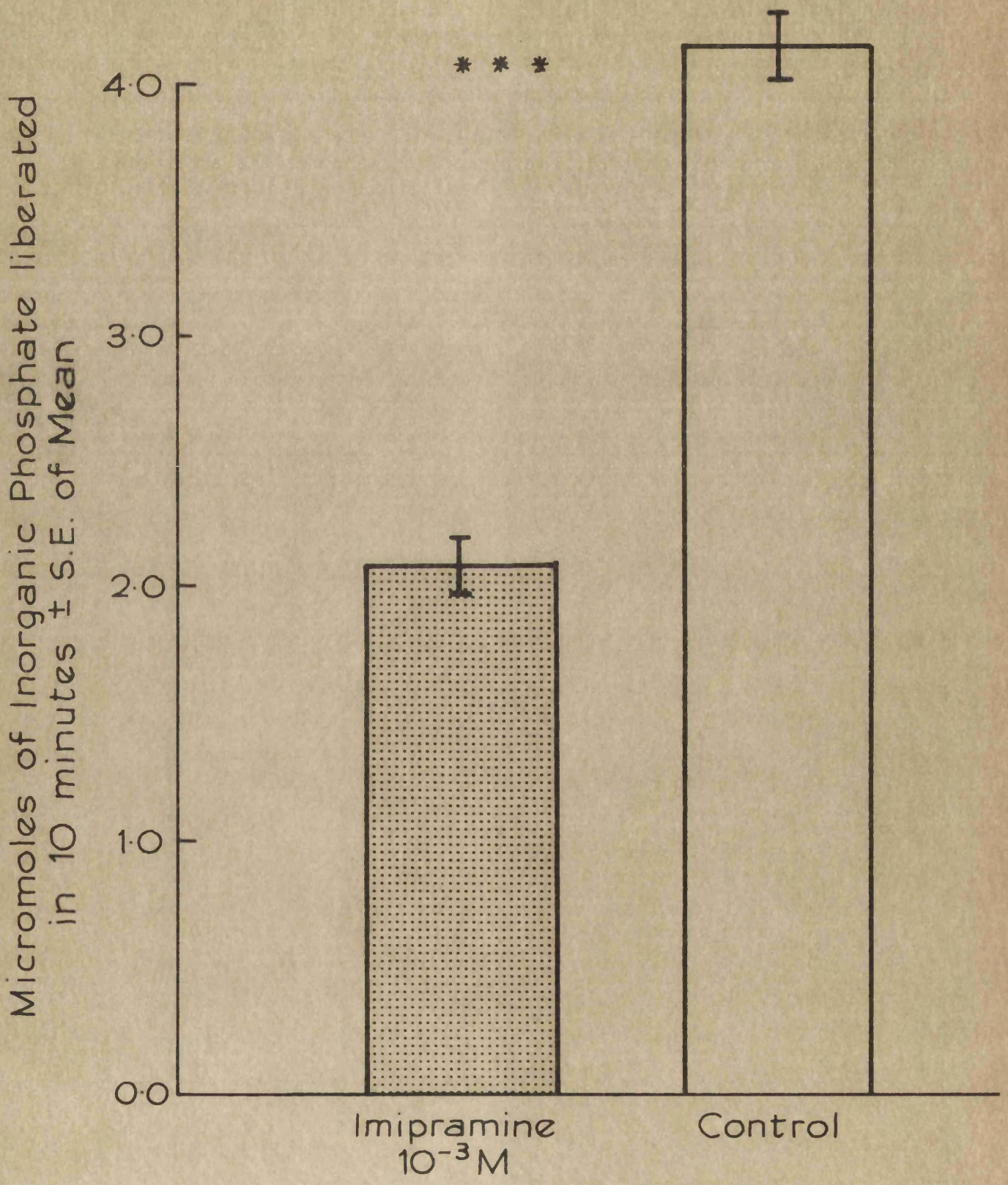
P < 0·001
* * *
Micromoles of Inorganic Phosphate liberated in 10 minutes ± S.E. of Mean
4·0
3·0
2·0
1·0
0·0
Imipramine 10^{-3}M
Control

Fig. XXXIX

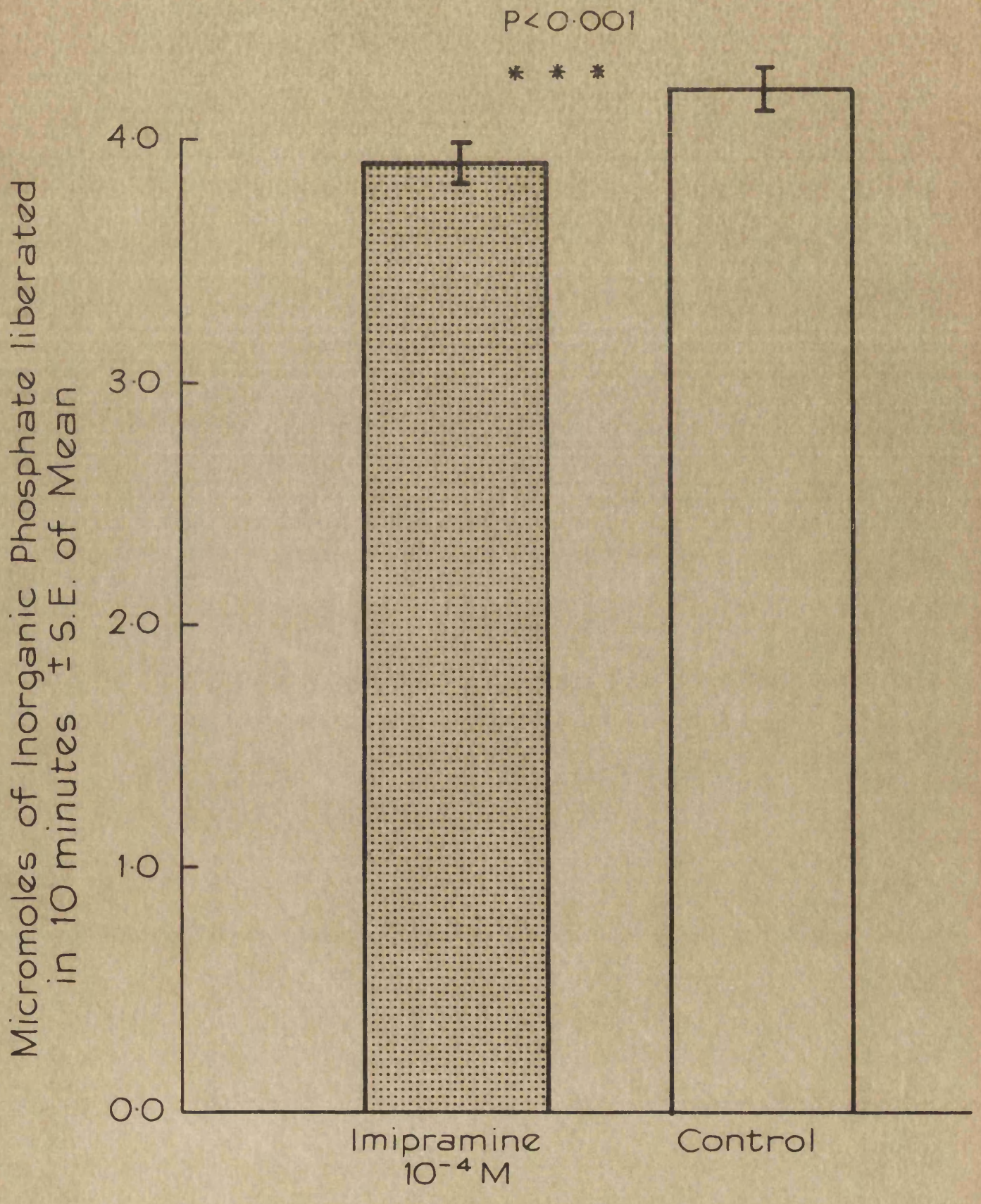
P<0·001
* * *
4·0
3·0
2·0
1·0
0·0
Micromoles of Inorganic Phosphate liberated in 10 minutes ± S.E. of Mean
Imipramine 10⁻⁴ M
Control

Fig. XL

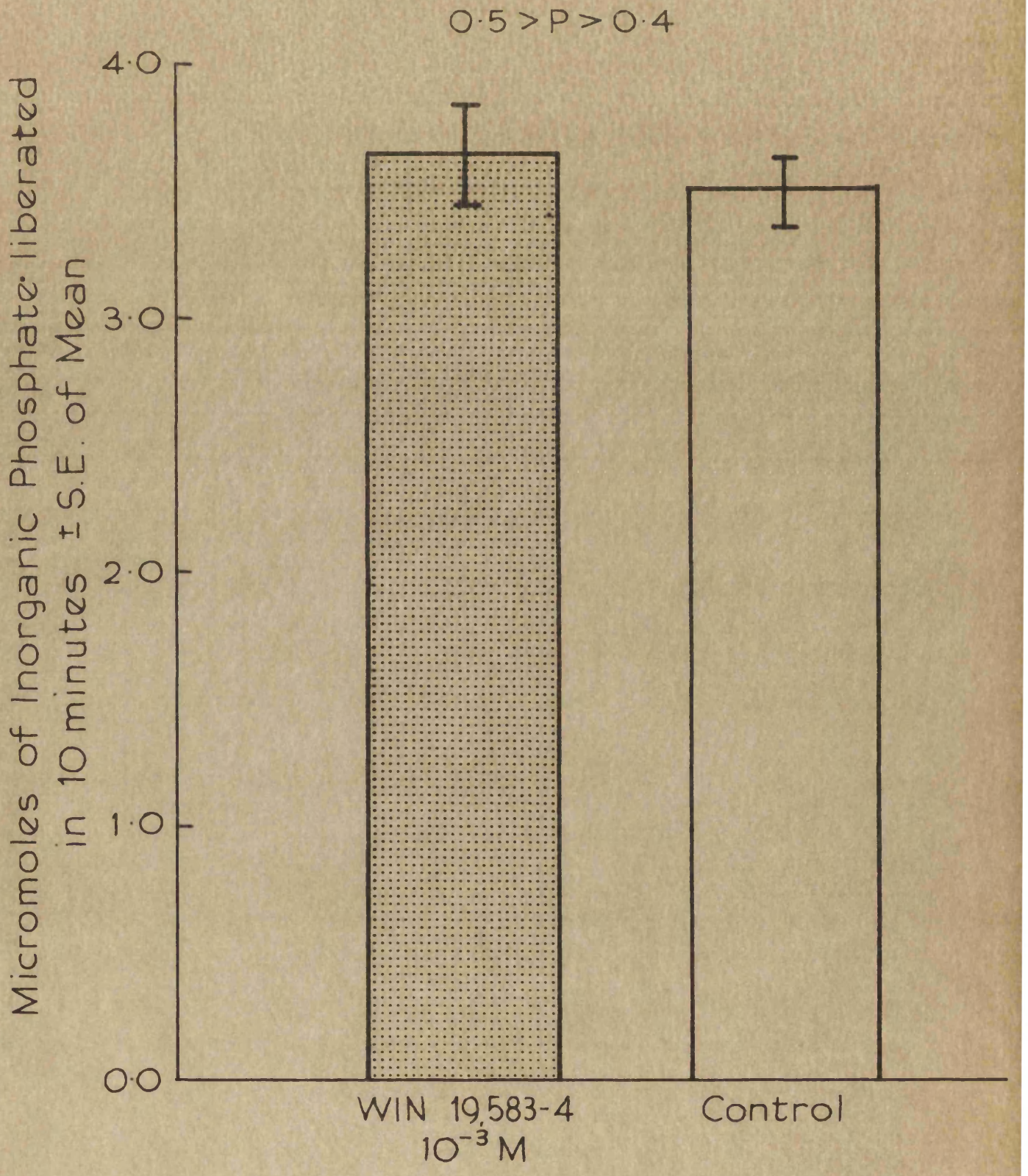
0·5 > P > 0·4
Micromoles of Inorganic Phosphate liberated in 10 minutes ± S.E. of Mean
4·0
3·0
2·0
1·0
0·0
WIN 19,583-4
10⁻³ M
Control

Fig. XLI

Table IV

Results of Respiration Experiments. Legend on Page 107.

Drug	Molar Concentration	First Hour	Second Hour	Third Hour	Fourth Hour	Fifth Hour
d-Amphetamine	Control	103.56 ± 5.66	93.73 ± 4.84	94.69 ± 2.82	101.82 ± 3.78	100.69 ± 5.21
	5×10^{-5} M	105.38 ± 4.96	92.49 ± 4.52	92.84 ± 1.64	96.33 ± 2.56	98.06 ± 5.50
		0.9>P>0.8	0.9>P>0.8	0.6>P>0.5	0.3>P>0.2	0.8>P>0.7
	5×10^{-4} M	99.48 ± 4.11	80.15 ± 4.74	51.27 ± 2.38	35.13 ± 3.33	25.65 ± 2.53
		0.6>P>0.5	0.1>P>0.05	P<0.001	P<0.001	P<0.001
l-Amphetamine	Control	92.20 ± 9.42	100.94 ± 1.44	114.89 ± 5.01	96.15 ± 5.04	98.22 ± 2.22
	5×10^{-4} M	82.39 ± 16.62	94.35 ± 3.92	103.11 ± 1.93	83.93 ± 2.96	71.41 ± 3.96
		0.7>P>0.6	0.2>P>0.1	0.1>P>0.05	0.1>P>0.05	0.01>P>0.001
	5×10^{-3} M	83.72 ± 15.93	72.14 ± 2.19	51.84 ± 3.31	22.93 ± 4.69	11.44 ± 5.48
		0.7>P>0.6	P<0.001	P<0.001	P<0.001	P<0.001
Methyl-Amphetamine	Control	107.57 ± 2.71	93.62 ± 2.36	98.92 ± 2.73	94.51 ± 2.55	84.29 ± 2.74
	5×10^{-5} M	105.95 ± 3.97	93.40 ± 1.37	96.78 ± 3.76	91.05 ± 2.45	80.57 ± 3.40
		0.8>P>0.7	P>0.9	0.7>P>0.6	0.4>P>0.3	0.5>P>0.4
	5×10^{-4} M	106.64 ± 2.15	94.15 ± 2.07	98.21 ± 2.12	84.78 ± 2.14	77.84 ± 2.50
		0.8>P>0.7	0.9>P>0.8	0.9>P>0.8	0.02>P>0.01	0.2>P>0.1

Table IV

Results of Respiration Experiments. (continued) Legend on Page 107.

Ephedrine	Control	93.19 - 2.97	70.22 - 2.19	88.95 - 3.07	88.33 - 4.24	76.22 - 5.11
	10^{-5} M	93.03 ± 3.68	76.65 ± 4.81	90.96 ± 4.85	90.80 ± 2.84	81.03 ± 2.44
		P > 0.9	0.3 > P > 0.2	0.8 > P > 0.7	0.7 > P > 0.6	0.5 > P > 0.4
	10^{-3} M	94.48 ± 5.55	74.99 ± 3.91	85.91 ± 5.16	88.00 ± 2.46	70.54 ± 3.58
		0.9 > P > 0.8	0.4 > P > 0.3	0.7 > P > 0.6	P > 0.9	0.4 > P > 0.3
Phenmetrazine	Control	104.82 ± 0.63	101.38 ± 1.37	108.21 ± 2.79	103.55 ± 3.00	116.07 ± 4.10
	10^{-5} M	106.25 ± 3.25	102.32 ± 2.68	107.82 ± 2.64	103.83 ± 2.05	111.46 ± 4.67
		0.7 > P > 0.6	0.8 > P > 0.7	P > 0.9	0.9 > P > 0.8	0.5 > P > 0.4
	10^{-3} M	109.20 ± 3.81	97.41 ± 2.57	98.54 ± 3.31	99.97 ± 2.63	109.42 ± 4.16
		0.3 > P > 0.2	0.2 > P > 0.1	0.05 > P > 0.02	0.4 > P > 0.3	0.3 > P > 0.2
WIN 19,583-4	Control	67.2 - 8.67	79.77 - 3.62	91.22 - 5.43	87.88 - 4.54	90.93 - 6.12
	0.8×10^{-5} M	68.47 ± 8.48	78.96 ± 3.10	91.37 ± 0.97	96.32 ± 7.42	84.13 ± 5.58
		P > 0.9	0.9 > P > 0.8	P > 0.9	0.4 > P > 0.3	0.5 > P > 0.4
	0.8×10^{-3} M	64.80 ± 13.67	69.13 ± 4.39	78.86 ± 5.48	78.20 ± 6.65	47.41 ± 7.34
		0.9 > P > 0.8	0.2 > P > 0.1	0.2 > P > 0.1	0.3 > P > 0.2	0.01 > P > 0.001

Table IV

Results of Respiration Experiments .(continued) Legend on Page 107.

Iproniazid	Control	109.70 ± 4.75	91.03 ± 4.01	96.40 ± 6.17	85.89 ± 5.24	60.96 ± 8.19
	10^{-5} M	108.44 ± 3.09	91.37 ± 2.69	93.32 ± 4.64	82.27 ± 4.90	58.78 ± 5.02
		0.9>P>0.8	P>0.9	0.7>P>0.6	0.7>P>0.6	0.9>P>0.8
	10^{-3} M	104.62 ± 2.99	92.86 ± 2.35	83.61 ± 5.58	67.73 ± 5.21	44.34 ± 7.02
		0.4>P>0.3	0.7>P>0.6	0.2>P>0.1	0.05>P>0.02	0.2>P>0.1
Isoniazid	Control	87.62 ± 5.00	97.86 ± 2.48	108.53 ± 3.13	112.90 ± 3.53	106.81 ± 4.69
	10^{-5} M	89.22 ± 6.19	93.68 ± 1.66	102.61 ± 1.83	107.81 ± 2.62	103.86 ± 5.45
		0.9>P>0.8	0.2>P>0.1	0.2>P>0.1	0.4>P>0.3	0.7>P>0.6
	10^{-3} M	90.80 ± 4.96	97.83 ± 1.74	104.21 ± 2.17	108.83 ± 2.25	107.01 ± 5.87
		0.7>P>0.6	P>0.9	0.3>P>0.2	0.4>P>0.3	P>0.9
Pheniprazine	Control	89.14 ± 4.80	87.57 ± 2.34	85.82 ± 2.43	100.32 ± 2.96	
	10^{-5} M	89.04 ± 6.03	83.30 ± 4.75	75.61 ± 9.05	84.64 ± 6.82	
		P>0.9	0.5>P>0.4	0.3>P>0.2	0.1>P>0.05	
	10^{-3}M	93.78 ± 4.17	45.57 ± 6.31	7.69 ± 2.22	11.67 ± 3.18	
		0.5>P>0.4	P<0.001	P<0.001	P<0.001	

Table IV

Results of Respiration Experiments. (continued) Legend on Page 107.

Imipramine	Control	90.44 ± 3.64	90.51 ± 1.29	99.46 ± 1.94	93.87 ± 4.13	104.56 ± 4.31
	4×10^{-4} M	91.70 ± 4.02	110.12 ± 2.71	87.67 ± 3.50	53.33 ± 4.22	41.11 ± 6.10
		0.9>P>0.8	P<0.001	0.02>P>0.01	P<0.001	P<0.001
	4×10^{-3} M	93.83 ± 2.43	13.74 ± 1.36	3.57 ± 1.33	3.08 ± 0.42	5.88 ± 1.16
		0.5>P>0.4	P<0.001	P<0.001	P<0.001	P<0.001
Amitriptyline	Control	93.28 ± 4.08	107.88 ± 4.21	89.04 ± 3.95	98.38 ± 6.00	105.91 ± 5.54
	4×10^{-4} M	95.11 ± 3.77	111.35 ± 4.52	47.77 ± 9.05	30.30 ± 5.82	21.09 ± 5.21
		P>0.9	0.6>P>0.5	0.01>P>0.001	P<0.001	P<0.001
	4×10^{-3} M	93.36 ± 5.09	25.97 ± 4.99	5.46 ± 1.85	6.51 ± 2.60	14.14 ± 4.94
		0.9>P>0.8	P<0.001	P<0.001	P<0.001	P<0.001
Sodium Barbitone	Control	77.19 ± 4.15	88.69 ± 2.03	99.00 ± 2.24	97.01 ± 2.26	85.83 ± 2.14
	10^{-3} M	79.48 ± 5.73	89.99 ± 2.54	95.88 ± 2.43	94.72 ± 1.47	88.77 ± 3.05
		0.8>P>0.7	0.7>P>0.6	0.3>P>0.2	0.5>P>0.4	0.5>P>0.4
	10^{-2} M	80.90 ± 5.31	88.96 ± 2.96	82.36 ± 3.13	67.02 ± 1.26	49.30 ± 2.92
		0.6>P>0.5	P>0.9	P<0.001	P<0.001	P<0.001

Table IV

Results of Respiration Experiments. (continued).

2,4-DNP	Control	93.4 ± 14.42	99.68 ± 3.85	100.18 ± 3.23	92.39 ± 10.14	
	2.5×10^{-5}M	109.17 ± 12.14	146.93 ± 5.10	150.27 ± 3.71	144.807 ± 1.87	
		0.5>P>0.4	P<0.001	P<0.001	0.1>P>0.001	

The Effects of a Number of Centrally-Acting Drugs and Some Chemically Related compounds Upon the In Vitro Respiration of Rat Brain Cerebral Cortex Slices.

The Results are in Microlitres of Oxygen Absorbed (± S.E. of Mean)/100 mg Wet Wt. Of Tissue/Hour.

The Significance of the Difference from the Control Mean in Each Experiment is given by the P value.

The Drug Solutions were tipped from the Sidearm into the Main Compartment at the End of the First Hour.

Table V

Drug	Molar Concentration	First Hour	Second Hour	Third Hour	Fourth Hour
Ephedrine	Control	158.16 ± 4.66	140.33 ± 2.26	113.01 ± 3.45	
	10^{-3} M	160.74 ± 2.58	132.33 ± 4.82	118.70 ± 3.02	
		0.7>P>0.6	0.2>P>0.1	0.2>P>0.1	
Phenmetrazine	Control	175.46 ± 2.48	163.45 ± 5.17	147.38 ± 2.69	129.67 ± 4.05
	10^{-3} M	179.20 ± 2.02	169.50 ± 3.20	151.82 ± 4.28	132.96 ± 3.89
		0.3>P>0.2	0.4>P>0.3	0.5>P>0.4	0.6>P>0.5
WIN 19,583-4	Control	199.56 ± 3.06	186.28 ± 6.02	130.885±10.55	
	10^{-4} M	200.59 ± 8.62	178.83 ± 4.91	127.88 ± 7.13	
		P>0.9	0.4>P>0.3	0.9>P>0.8	
WIN 19,583-4	Control	187.80 ± 5.10	158.59 ± 7.06	140.55 ± 4.52	87.89 ± 8.09
	10^{-3} M	197.15 ± 3.28	152.48 ± 4.72	94.86 ± 7.40	39.72 ± 3.26
		0.2>P>0.1	0.5>P>0.4	P<0.001	P<0.001
Pheniprazine	Control	181.47 ±10.08	181.81 ± 9.44	113.34 ± 5.60	96.26 ± 3.69
	10^{-4} M	187.88 ± 4.61	149.84 ± 5.97	54.74 ± 5.57	42.48 ± 2.79
		0.6>P>0.5	0.02>P>0.01	P<0.001	P<0.001
Isoniazid	Control	211.13 ± 4.23	207.19 ± 5.43	175.97 ± 2.16	142.01 ± 5.29
	10^{-3} M	204.81 ± 6.18	194.21 ±10.49	162.78 ± 2.59	99.44 ± 5.28
		0.5>P>0.4	0.4>P>0.3	0.01>P>0.001	P<0.001
Amitriptyline	Control	150.66 ± 1.74	131.30 ± 3.16	82.49 ± 3.36	53.87 ± 2.57
	4×10^{-4} M	92.17 ± 5.44	50.48 ± 2.48	24.92 ± 2.32	24.09 ± 2.79
		P<0.001	P<0.001	P<0.001	P<0.001
Sodium Barbitone	Control	146.13 ± 2.26	139.26 ± 3.39	115.05 ± 3.85	
	5×10^{-4} M	137.86 ± 2.48	135.49 ± 2.72	118.98 ± 4.94	
		0.05>P>0.02	0.5>P>0.4	0.6>P>0.5	

Results of 2,4-Dinitrophenol-Stimulated Respiration Experiments.

The Effects of a Number of Centrally-Acting Drugs and some Chemically Related Compounds Upon the In Vitro 2,4-Dinitrophenol-stimulated Respiration of Rat Brain Cerebral Cortex slices.

The Results are in Microlitres of Oxygen Absorbed (± S.E. of Mean)/100 mg Wet Wt. of Tissue/Hour.

The Significance of the Difference from the Control Mean in Each Experiment is given by the P value.

The Drug Solutions were tipped from the Sidearm into the Main Compartment at the Beginning of the Experiment.

DISCUSSION

DISCUSSION

The use of *in vitro* techniques in investigating the modes of action of drugs is based upon the fundamental assumption that the biochemical reactions which occur in the intact animal also take place to some extent in isolated tissue slices, tissue homogenates and other cellular fractions (Quastel, 1957). While *in vitro* studies have been especially valuable in the field of drug-enzyme interactions, the results of these experiments must be interpreted with caution. Thus, before the *in vivo* pharmacodynamic activity of a drug can be explained on the basis of an *in vitro* effect on a particular biochemical mechanism, it is necessary to establish whether or not this same mechanism is disturbed *in vivo* and indeed, whether such an interference could quantitatively explain the activity of the drug (Hunter & Lowry, 1956). Furthermore, the concentration of drug producing the *in vitro* effect must be no greater than that required to produce the *in vivo* pharmacodynamic effect. On the other hand, the inability of a drug to inhibit an enzyme, *in vitro*,

does not prove conclusively that the enzyme is unaffected *in vivo* (Hunter *et al*, 1956). However, despite these limitations, *in vitro* studies are very useful in the investigation of the modes of action of centrally-acting drugs, since they provide a means by which the biochemical aspects of the activity of these drugs can be examined in some detail.

The intact normal human brain derives its energy almost entirely from the aerobic oxidation of glucose (Kety, 1957). Alteration in the neuronal activity of the brain *in vivo* is usually associated with a modification of cerebral oxygen uptake. For example, the depression of cerebral respiration during diabetic coma (Kety, Polis, Nadler & Schmidt, 1948), thiopentone anaesthesia (Wechsler, Dripps & Kety, 1951) and acute alcohol intoxication (Battey, Heyman & Patterson, 1953) is accompanied by a significant reduction in the oxygen uptake. A number of alterations in mental function brought about by the administration of such drugs as chlorpromazine (Fazekas, Albert & Alman, 1955) and lysergic acid (Kety, 1957) cannot be related to changes

in the rate of respiration. It is possible that biochemical changes responsible for the pharmacodynamic effects of centrally-acting drugs, might be confined to a particular area or areas within the brain, so small as to have no effect upon the overall oxygen uptake (Kety, 1957). It is also possible that the primary biochemical sites of action may be mechanisms involved in the utilisation rather than in the production of energy.

The results obtained in the present investigation indicate that the centrally-acting drugs studied do not act primarily by modifying the cerebral oxygen uptake. Thus, a number of central nervous system stimulants including d-amphetamine, methylamphetamine, iproniazid and pheniprazine, all of which significantly increase the rat brain levels of adenosinetriphosphate (ATP), lower the levels of adenosinediphosphate (ADP) and increase the ATP/ADP ratio (Van Petten & Lewis, 1962; Lewis & Van Petten, 1962), had no effect on the *in vitro* oxygen uptake at concentrations corresponding to those likely to be found in the brain following the administration of therapeutic doses. At very high

concentrations, however, these compounds inhibited respiration markedly. Similar effects were obtained in the experiments using 2,4-dinitrophenol-stimulated respiration. In this case, the inhibition of oxygen uptake produced by the central nervous system stimulant drugs was more pronounced.

When these results are considered in the light of the hypothesis that a wide variety of central stimulant drugs may produce their characteristic pharmacodynamic effects by virtue of an ability to increase the synthesis of adenosinetriphosphate within the brain (Van Petten, 1962), it is clear that they do not support such a view. Although the results of this investigation suggest that an alteration in the balance between energy production and energy utilization as shown by the increased ATP/ADP ratio, cannot be explained on the basis of an increased respiration, they do not prove this.

While the function of noradrenaline and 5-hydroxytryptamine within the brain have not yet been established, it is possible that these amines may have a neurohumoral function in certain areas concerned in

the activity of central stimulant drugs. The ability of such drugs as iproniazid and tranylcypromine to inhibit the enzyme monoamine oxidase, which is partly responsible for the inactivation of these amines, is believed to be closely related to their ability to produce central stimulation. Among the drugs studied in this investigation, the most potent inhibitors of rat brain mitochondrial monoamine oxidase activity were the hydrazine derivatives, tranylcypromine and iproniazid. This study also confirmed the comparatively much lower activity of the amphetamines and other related phenylethylamine derivatives. Thus, in the case of d-amphetamine and methylamphetamine, the inhibition of monoamine oxidase is probably of little importance in mediating their pharmacodynamic effects, since they are almost equipotent with l-amphetamine which is a much weaker central nervous stimulant. Although imipramine and the closely related iminodibenzyl derivative, amitriptyline, were approximately one hundred times less active than iproniazid, they were more potent than the amphetamine-like compounds. This finding is of interest, since, although other

workers have reported that imipramine is not a potent inhibitor of monoamine oxidase (Pletscher & Gey, 1959; Pulver, Exer & Herrman, 1960), a number of observations have indicated the possibility of a relationship existing between its mechanism of action and amine metabolism. Thus, this view is supported by the discovery that imipramine enhances and prolongs the effects of exogenously supplied noradrenaline upon the cardiovascular system and the nictitating membrane (Sigg, 1959; Schaeppi, 1960). Whether this aspect of the activity of imipramine is of importance with respect to the central activity of this drug is a problem requiring further investigation.

Although the role of acetylcholine in the brain and spinal cord has not been completely elucidated, it is probable that it has a transmitter function in certain areas, including the optic nerve pathway (Feldberg et al, 1948) and the Renshaw cells of the spinal cord (Eccles et al, 1956). Acetylcholine has also been postulated to act at the mesodiencephalic level (Feldberg, 1957) and it has been suggested that the reticular activating system might contain both

cholinergic and adrenergic components (Magoun, 1958).

The administration of acetylcholine initiates in the EEG the appearance of fast, low voltage waves similar to those observed in an alerted animal. Furthermore, high doses of acetylcholine produce EEG patterns characteristic of a convulsive discharge (Feldberg, 1957). Similar effects are produced following the administration of potent anti-acetylcholinesterases such as eserine and diisopropylfluorophosphonate (Rinaldi & Himwich, 1955). The EEG desynchrony produced by acetylcholine is not associated, however, with behavioural arousal. Moreover, while the potent anticholinergic, atropine, produces an EEG pattern characteristic of sleep, it does not cause behavioural sleep (Bradley et al, 1957).

There is a similarity in the effects on the EEG produced by acetylcholine and the central nervous system stimulant, amphetamine, both of which produce EEG desynchrony by an action on the reticular formation of the brain (Feldberg, 1957) (see pages 6-I2). However while the EEG arousal induced by both

acetylcholine and amphetamine can be abolished by the administration of atropine, the behaviour of the animal is unaffected. An interpretation of this dissociation of EEG and behavioural responses is possible if it is accepted that the reticular formation contains both adrenergic and cholinergic components (Magoun, 1958). Thus the amphetamine-like central stimulants may produce their characteristic pharmacodynamic effect by a direct action on the adrenergic component of the reticular formation and may initiate EEG alerting through some other indirect action on the cholinergic component. In these circumstances atropine would abolish the EEG activation without interfering with the behavioural effect. Furthefmore, the EEG desynchrony produced by acetylcholine and anti-acetylcholinesterases would not be associated with behavioural arousal if these drugs had no effect upon the adrenergic component of the reticular formation.

From this present investigation it is apparent that amphetamine and other chemically closely related compounds have only very weak anti-acetylcholinesterase activity. It therefore is unlikely that the hypothetical

indirect action of amphetamine on the cholinergic component of the reticular formation can be explained on the basis of the inhibition of this enzyme. In the case of the hydrazine derivatives, pheniprazine and iproniazid, which were slightly more potent, it also seems unlikely that the inhibition of this enzyme plays an important part in mediating their pharmacological effects. Although imipramine and amitriptyline were the most potent inhibitors of acetylcholinesterase in the present investigation, their activities were much lower than would be required if this property were to be regarded as an important factor in the central activities of these drugs.

In order that the pharmacological significance of the effects of the centrally-acting drugs studied, on adenosinetriphosphatase, might be discussed, it is necessary to consider both the functional importance of this enzyme in neural tissues and the biochemical role of its substrate, adenosinetriphosphate. The hydrolysis of adenosinetriphosphate within the brain, to form adenosinediphosphate and inorganic phosphate, provides the energy necessary for a number of chemical syntheses including that of glutamine (McIlwain, 1957), noradrenaline (Kirshner, 1959, 1960) and acetylcholine (McIlwain, 1959). Adenosinetriphosphate is also concerned in the binding of catecholamines in the adrenal medulla (Hillarp, 1960). Most of the energy made available by the breakdown of this compound is, however, required by the brain for the maintainance of ionic gradients (Richter & Crossland, 1949; Richter, 1952; Heald, 1960, McIlwain, 1957, 1962). Suggestive evidence supporting the view that adenosinetriphosphatase is involved as a link between metabolism and the active transport of ions across cell membranes has been provided by a number of

experiments. Thus, it has been shown that the potassium influx into human red blood corpuscles is linearly related to the amount of adenosinetriphosphate hydrolysed in the same time (Dunham, 1957; Whittam, 1958). Furthermore, adenosinetriphosphate supports the active efflux of sodium ions, a response which does not occur with inosine triphosphate or adenosinediphosphate (Hoffman, 1960). Studies on the effects of anoxia (Shares & Berman, 1955), metabolic inhibitors (Hodgkin & Keynes, 1955) and 2,4-dinitrophenol (Caldwell, 1960; Caldwell & Keynes, 1957), upon both the levels of adenosinetriphosphate and the flux of sodium and potassium ions in the giant axon of the squid, have also indicated that adenosinetriphosphate may be concerned in the active transport of these ions in neural tissue.

A number of investigations have been undertaken to determine whether or not the enzyme, adenosine-triphosphatase, might also be implicated in the ion transport process (Whittam, 1961). Adenosine-triphosphatase activity has been reported in a number of neural tissues, including the sheathes of giant nerve

axons (Libet, 1948; Abood & Gerard, 1954) and the peripheral nerves of the rat (Abood et al, 1954). Several kinds of adenosinetriphosphatases are known to exist. Thus, the nerves of the shore crab contain two adenosinetriphosphatases, which may be differentiated on the basis of their activation either by calcium or magnesium ions (Skou, 1957). An adenosinetriphosphatase, isolated from muscle was, however, stimulated by magnesium ions and inhibited by calcium ions (Kielley & Meyerhoff, 1948). The magnesium activation of crab nerve adenosinetriphosphatase was increased in the presence of sodium ions and still further increased by the concomitant addition of small concentrations of potassium ions (Skou, 1957). A similar effect was observed using a preparation of brain adenosine-triphosphatase (Hess & Pope, 1957). Generally, the presence of magnesium ions seems to be necessary for the activity of the enzyme and for its further activation by sodium and potassium ions (Whittam, 1961). While the effect of calcium ions appears to be almost entirely inhibitory, the effect of potassium ions is dependent upon the concentration used. Thus, high concentrations

of potassium ions prevent the sodium-activation of adenosinetriphosphatase but have no effect upon the magnesium-activation of the enzyme (Whittam, 1961).

The many similarities between the mechanism of hydrolysis of adenosinetriphosphate and the active transport process have been discussed by Whittam (1961). These include a similar location in the neural membrane and a similarity between the concentrations at which potassium and ammonium ions produce half their maximal effects in these two processes. Furthermore, the possibility of the existence of a relationship is strengthened by the finding that strophanthin, which is an inhibitor of active ion transport, prevents both the sodium and the combined sodium and potassium-activation of adenosinetriphosphatase (Skou, 1960).

As a result of the possible involvement of adenosinetriphosphatase and adenosinetriphosphate in neural energy metabolism and in the mechanism of active transport, a number of investigations have been carried out to determine the effects of centrally-acting drugs on the activity of this enzyme and on the brain levels of its substrate. The modification of the activity

of adenosinetriphosphatase would provide a very convenient means of explaining the pharmacodynamic effects of such drugs as reserpine and chlorpromazine (Albaum & Milch, 1962). Thus, if a drug-induced, *in vivo* inhibition of brain adenosinetriphosphatase activity could be demonstrated, the accumulation of substrate, by affecting the energy metabolism, might eventually result in a decreased synthesis of adenosinetriphosphate (Albaum *et al*, 1962). However, while results indicating that chlorpromazine causes a temporary rise in the brain levels of adenosine-triphosphate, followed by a sustained fall, might be interpreted on the basis of an effect upon some homeostatic mechanism, responsible for the control of the synthesis and utilisation of this compound, the nature of the role of adenosinetriphosphatase in this system remains unexplained (Albaum *et al*, 1962). On the other hand, if the possible existence of such a homeostatic mechanism is ignored, the results of experiments in which it was shown that a variety of central stimulant drugs, increased the brain levels of adenosinetriphosphate, might be explained on the basis

of the *in vivo* inhibition of adenosinetriphosphatase.

The results obtained in this investigation indicate that the inhibition of adenosinetriphosphatase activity is unlikely to explain the increased brain levels of adenosinetriphosphate (Van Petten & Lewis, 1962; Lewis & Van Petten, 1962), produced by the amphetamines and tranylcypromine. Thus, whereas tranylcypromine had no effect on the *in vitro* activity of adenosinetriphosphatase, *d*-amphetamine produced a significant effect at the very high concentration of 10^{-2} M. In contrast, imipramine (10^{-3} M, 10^{-4} M) produced a very marked inhibition at a dose, which, although high, was much nearer to a concentration likely to be found, *in vivo*, following the administration of a therapeutic dose of the drug.

Little correlation exists between the inhibitory effects of the central nervous system stimulant drugs studied, on *in vitro* brain adenosinetriphosphatase activity and their ability to increase the brain levels of adenosinetriphosphate. Thus, imipramine is a more potent inhibitor of this enzyme than *d*-amphetamine, which increases the brain levels of adenosinetriphosphate

more markedly. These results are generally in agreement with the *in vivo* observations of Lu and Krantz (1953) who showed that *d*-amphetamine and cocaine had only a very slight inhibitory effect on the adenosinetriphosphatase activity of the rat brain. It cannot be concluded however, that the mechanism of action of the central nervous system stimulant drugs studied, is unrelated to the modification of the activity of this enzyme *in vivo*, because of the difficulty in interpreting the results of *in vitro* estimations of adenosinetriphosphatase activity, which is profoundly affected even by varying the relative concentrations of the cations and anions in the reaction system (Umbreit *et al*, 1957). Thus, it is possible that if a reaction system containing different concentrations of ions were to be used, the central nervous system stimulant drugs studied might produce quite different effects.

SUMMARY AND CONCLUSIONS

An investigation has been made of the effects of some central nervous system stimulants including phenylethylamine, hydrazine and iminodibenzyl derivatives (Table VI page I93-5) and a number of chemically related compounds upon the normal and 2,4-dinitrophenol-stimulated oxygen uptake of slices of rat brain cerebral cortex and upon the activity of the acetylcholinesterase, monoamine oxidase and adenosinetriphosphatase present in rat brain homogenates and mitochondrial preparations.

The more potent central nervous system stimulants generally inhibited the oxygen uptake when used at a sufficiently high concentration. The dose levels required to produce an effect were very much higher than those likely to be found in the brain following the administration of therapeutic doses. On the other hand, weaker central nervous system stimulants (e.g. phenmetrazine and isoniazid) had no effect on respiration even at very high concentrations. Exceptions were found in the cases of l-amphetamine, which was a potent inhibitor of oxygen uptake and in the

case of the potent central nervous system stimulant, iproniazid which had only a very slight effect on *in vitro* respiration. The central nervous system depressant, sodium barbitone also inhibited the *in vitro* oxygen uptake at high dose levels. Of all the centrally-acting drugs studied, only imipramine increased the oxygen uptake. This effect, which was only observed in the second hour of the experiment, using a low dose, was subsequently reversed. The stimulation of respiration produced by imipramine, although significant, was neither as marked nor as well sustained as that produced by the potent uncoupling agent, 2,4-dinitrophenol.

Using 2,4-dinitrophenol-stimulated respiration, the weaker central stimulants had little or no effect on oxygen uptake but drugs, previously found to have only a very slight inhibitory effect on unstimulated respiration, now had a very much more marked effect.

The potent monoamine oxidase inhibitory activity of the central stimulants, iproniazid and tranylcypromine was confirmed. In the case of the

amphetamines, which were less active in this respect than the hydrazine derivatives, it is likely that they do not owe their central stimulant activity to an ability to inhibit this enzyme. This view is supported by the fact that although d-amphetamine has considerably greater central stimulant activity than l-amphetamine, the two stereoisomers were found to be almost equally active inhibitors of rat brain mitochondrial monoamine oxidase activity. Imipramine and amitriptyline were more potent inhibitors of this enzyme than the amphetamines. The relative importance of this property in explaining the central stimulant activities of the iminodibenzyl derivatives is a problem requiring further investigation.

The results of the acetylcholinesterase experiments indicate that the inhibition of this enzyme by central stimulant drugs is probably not an important factor in the central activity of these compounds. Although imipramine and d-amphetamine, when used at high dose levels, significantly inhibited the adenosinetriphosphatase activity of rat brain

homogenates, no correlation has been shown to exist between these effects and the pharmacodynamic activities of these drugs. Since, however, the effects of central nervous stimulant drugs on this enzyme system might be more marked *in vivo* it cannot as yet be concluded that the mechanisms of action of these compounds are not mediated through an effect on adenosinetriphosphatase.

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APPENDICES

APPENDIX I

The preparation of reagents and saline solutions

With the exception of the sodium metabisulphite and 1-amino-2-napthol-4-sulphonic acid, the chemicals used in this investigation were of 'Analar' grade. Only deionised water (conductivity 1.5×10^{-5} ohms/cm) was used to prepare the solutions.

Acetylcholine chloride, (0.063 M) 0.5722 g Roche $(CH_3)_3NClCH_2CH_2OCOCH_3$ was dissolved in 50 ml of water. The contents of 5 ampoules (each containing 100 mg) were dissolved in water and transferred to a 50 ml flask. The contents of another ampoule were dissolved in water and transferred to a 10 ml flask which was then adjusted to volume. 7.2 ml of this solution were transferred to the original 50 ml flask which was finally adjusted to volume with water. The solution was stored at $4^{\circ}C$.

Adenosine Triphosphate, (0.02 M) 0.1213 g Sigma $ATP.2Na.3H_2O$ was dissolved in 7.0 ml ice-cold water. The pH of the solution was adjusted to 7.2 with normal sodium hydroxide. The volume was made up to 10 ml with ice-cold water. 5 x 2.0 ml aliquots were frozen and stored at $-15^{\circ}C$.

Calcium Chloride, (0.02 M) 0.2220 g British Drug Houses $CaCl_2$, which had been dried in an oven at 150°C for 12 hours, was dissolved in water and the volume adjusted to 100 ml. The solution was stored at 4°C.

Control Solutions, a suitable control solution for each of the drugs to be investigated was prepared by adjusting the pH of deionized water to that of the drug solution by means of the appropriate acid.

Copper Sulphate, (0.02 M) 4.9938 g British Drug Houses $CuSO_4.5H_2O$ were dissolved in water and the volume adjusted with water to 1000 ml.

2,4-Dinitrophenol, (final concentration 2.5×10^{-5}M). The solution was prepared so that 0.3 ml diluted to 3.0 ml gave a final concentration of 2.5×10^{-5}M. 0.4603 g British Drug Houses $C_6H_4.N_2O_5$ was dissolved in water and the volume adjusted to 100 ml with water. This solution was diluted (1 in 100) with Krebs' Ringer phosphate medium containing 0.2% (w/v) glucose. The solution was stored at 4°C.

Fiske and Subarrow Mixture (F.S.M.)

(a) Ammonium Molybdate Solution 2.5 g British Drug Houses $(NH_4)_6MO_7O_{24}.4H_2O$ were dissolved in water and the volume was adjusted to 100 ml with water.

(b) Reducing Reagent 2.4 g British Drug Houses sodium sulphite, $Na_2SO_3.7H_2O$ and 10.9616 g British Drug Houses 'Reagent' grade sodium metabisulphite $Na_2S_2O_5$ were dissolved in about 90 ml of water. 0.20 g British Drug Houses 'Reagent' grade 1-amino-2-Napthol-4-sulphonic acid, $NH_2C_{10}H_5(OH)SO_3H$ was added to this solution and the mixture shaken using a mechanical shaker for 30 minutes, to effect solution. The volume was adjusted to 100 ml with water. Finally the solution was filtered.

Both solutions were stored at 4°C. F.S.M. was prepared by combining 2 volumes of solution (a) with 1 volume of solution (b). The resulting solution was maintained at 0°C and was used for not longer than 1 day.

Glycylglycine Buffer, (0.3 M, pH 7.4) 3.9636 g British Drug Houses $NA_2CH_2COCONHCH_2COOH$ were dissolved in 60 ml of water. The pH was adjusted to 7.4 by the addition of normal sodium hydroxide (approximately 5 ml). Finally the solution was diluted to 100 ml with water and stored at 4°C.

Isotonic saline, (0.9% w/v) 9.0 g British Drug Houses NaCl were dissolved in water and the volume adjusted to 1000 ml with water. The solution was stored at 4°C.

Magnesium Chloride, (0.02 M) 0.4067 g British Drug Houses $MgCl_2.6H_2O$ was dissolved in water and the volume adjusted to 100 ml. The solution was stored at 4°C.

Perchloric Acid, (1.0 M) 8.21 ml British Drug Houses $HClO_4$ (71-73% w/w) were added to about 90 ml of water. The solution was adjusted to 100 ml by the addition of water and was stored at 4°C.

Perchloric Acid, (0.05 M) 4.15 ml British Drug Houses $HClO_4$ (71-73% w/w) were added to about 900 ml of water. The solution was adjusted to 1000 ml by the addition of water and was stored at 4°C.

Potassium Chloride, (1.0 M) 7.455 g British Drug Houses KCl were dissolved in water and the volume adjusted to 100 ml by the addition of water. The solution was stored at 4°C.

Potassium Hydroxide, (20% w/v) 20.0 g British Drug Houses KOH were dissolved in water and the volume adjusted to 100 ml.

Physiological Saline Solutions

Salts (g/litre)	Krebs' Ringer Phosphate (Modified)	Krebs' Ringer Bicarbonate (Modified)
Sodium Chloride	7.03	7.03
Potassium Chloride	0.358	0.358
Calcium Chloride (anhydrous)	0.40	0.40
Sodium Phosphate (dihydrate)	2.78	
Magnesium Sulphate (heptahydrate)	0.296	0.296
Sodium Bicarbonate		2.34
Glucose	2.0	2.0

Krebs' Ringer-bicarbonate (modified) was gassed with a mixture of carbon dioxide (5%) and nitrogen (95%) for a period of 10 minutes before use.

Sodium Cyanide, (final concentration 0.0033 M). The solution was prepared so that 0.4 ml diluted to 3.0 ml produced a final concentration of 0.0033 M,. 0.1214 g British Drug Houses NaCN was dissolved in water and the volume adjusted to 100 ml. The solution was stored at 4°C.

Sodium Hydroxide, (normal) British Drug Houses volumetric solution of NaOH was used.

Sorensen's Phosphate Buffer, (0.067 M, pH 7.2) 0.2400 g sodium dihydrogen phosphate British Drug Houses NaH_2PO_4 (anhydrous) and 0.6629 g British Drug Houses disodium hydrogen phosphate Na_2HPO_4 (anhydrous) were dissolved in water and the volume adjusted to 100 ml. The solution was stored at 4°C.

Standard Inorganic Phosphate, (final concentration 1 micromole/ml). 0.1361 g British Drug Houses potassium dihydrogen phosphate KH_2PO_4 was dissolved in 0.05 M perchloric acid and adjusted to 100 ml. This solution was diluted (1 in 10) to give a solution containing 1 micromole/ml. A number of solutions ranging from 0.25 micromoles/25 ml to 6 micromoles/25 ml were prepared using 0.05 M perchloric acid.

Sucrose, (0.25 M) 21.394 g British Drug Houses $C_{12}H_{22}$ were dissolved in water and the volume adjusted to 250 ml. This solution was stored at 4°C.

Tyramine Hydrochloride, (final concentration 0.01 M). This solution was prepared so that 0.2 ml diluted to 3.0 ml produced a final concentration of 0.01 M. 2.5977 g of Light & Co. $C_8H_{11}NO.HCl$ were dissolved in water and the volume adjusted to 20 ml. The solution was stored at 4°C.

APPENDIX II

Statistics

Student's '<u>t</u>' test was used to determine the significance of the difference between the means of test and control experiments. The calculations were made using the following basic equations.

The <u>variance</u> (S^2) from the mean ($\bar{x}$) for each set of variates was calculated by the equation.

$$S^2 = \frac{x^2 - \frac{(\Sigma x)^2}{n}}{n - 1}$$

where 'n' is the number of variates.

The <u>Standard Deviation</u> ($\overline{S.D.}$) from the mean for two sets of variates was calculated by the equation.

$$\overline{S.D.}^2 = \frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}$$

The values of '<u>t</u>' were then obtained from the following equation.

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\overline{S.D.}}$$

The value of P was obtained from tables. The number of degrees of freedom (D.f.) were calculated from the following expression.

$$D.f. = (n_1 - n_2 - 2)$$

Sample calculation

Drug treated	Control
1.965	3.835
2.040	3.985
1.915	4.195
1.850	4.575
2.405	3.725
2.410	4.700
2.410	3.640
2.290	4.140
2.000	4.460
1.595	4.080

Symbol	Drug treated	Control
x	20.880	41.335
x^2	44.3009	172.0089
$(x)^2/n$	43.5974	170.8582
S^2	0.07817	0.12786
S.D. $(\sqrt{S^2})$	0.2796	0.3575
S.E.M. $(\frac{S.D.}{n})$	0.08842	0.11305
$\overline{S.D.}^2$	0.02061	
$\overline{S.D.}$	0.01436	
t	142.409	
D.f	18	
P	0.001	

There is therefore a highly significant difference between the means of test and control experiments.

In the histograms in this thesis, significance is denoted in the following manner.

*	$0.05 > P > 0.01$	significant.
**	$0.01 > P > 0.001$	very significant.
***	$P < 0.001$	very highly significant.

Regression Line

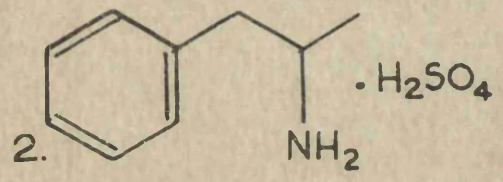
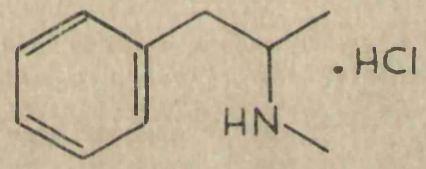
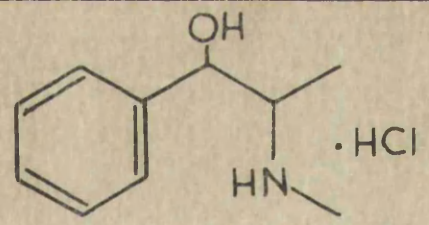
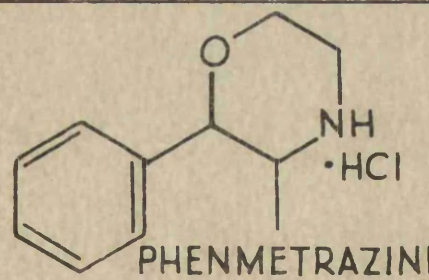
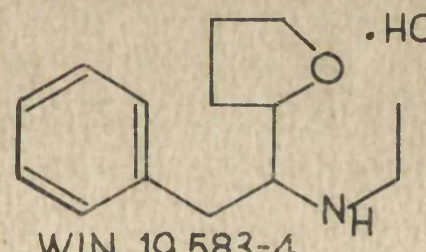
The points required for drawing the regression line were calculated from the following formulae.

$$b = \frac{(xy) - \bar{x}\, y}{x^2 - \bar{x}\, x}$$

$$a = \bar{y} - b\bar{x}$$

where $\bar{x}$ and $\bar{y}$ are the mean values of the two variables and where 'a' gives the point on the vertical axis through which the line must pass when b = 0 (Fig. VI , Page 59)

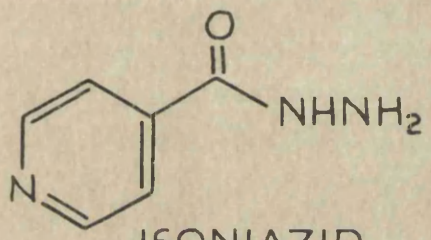
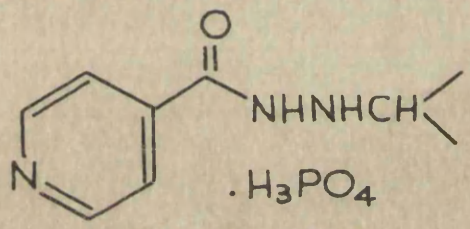
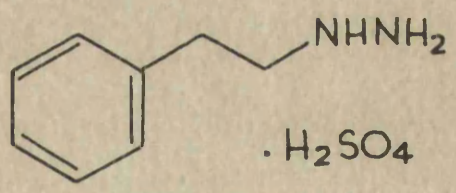
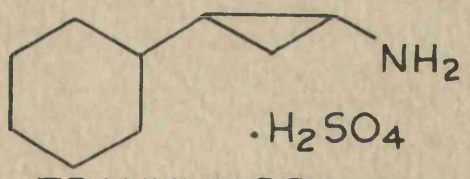
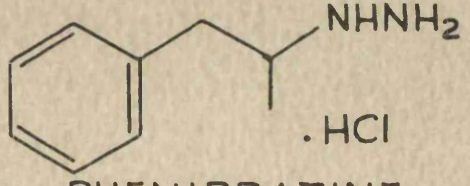
Table VI

Structural formula and approved name	Enantiomorph used	Source
 2. $\cdot H_2SO_4$ NH_2 AMPHETAMINE	*dextro* *laevo*	Smith, Kline & French Laboratories Ltd.
 $\cdot$HCl HN METHYLAMPHETAMINE	*dextro*	Burroughs Wellcome & Co.
 OH $\cdot$HCl HN EPHEDRINE	*laevo*	British Drug Houses Ltd.
 O NH $\cdot$HCl PHENMETRAZINE	*racemate*	Pfizer Ltd.
 $\cdot$HCl O NH WIN 19,583-4	*racemate*	Bayer Products Division of Winthrop Group Ltd.

(a) Amphetamine-like compounds.

Drugs Employed.

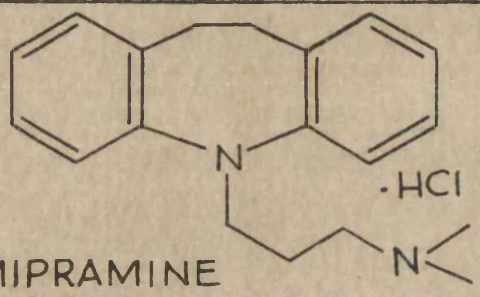
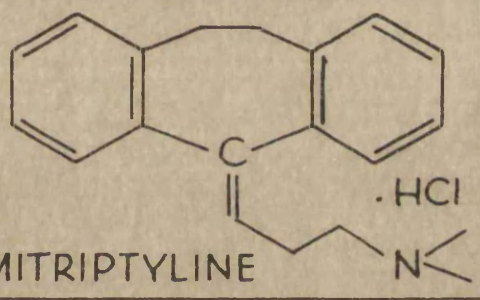
Table VI

Structural formula and approved name	Enantiomorph used	Source
 ISONIAZID		Smith and Nephew Research Ltd.
 IPRONIAZID		Roche Products Ltd.
 PHENELZINE		William R. Warner & Co. Ltd.
 TRANYLCYPROMINE	*racemate*	Smith, Kline and French Laboratories Ltd.
 PHENIPRAZINE	*racemate*	Benger Laboratories Ltd.

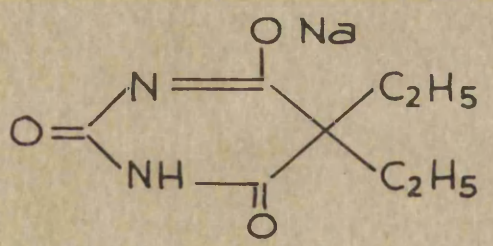
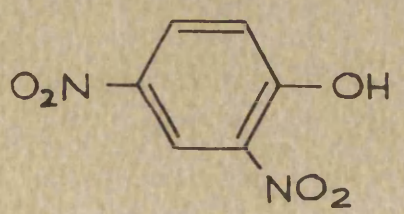
(b) Hydrazine derivatives and related compounds.

Drugs Employed.

Table VI

Structural formula and approved name	Enantiomorph used	Source
 IMIPRAMINE		Geigy Pharmaceutical Co. Ltd.
 AMITRIPTYLINE		Merck Sharpe and Dohme Ltd.

(c) Iminodibenzyl derivatives

Structural formula and approved name	Enantiomorph used	Source
 BARBITONE SODIUM		
 2,4 DNP		British Drug Houses Ltd.

(d) Miscellaneous compounds

Drugs Employed.

SUMMARY

The Effects Of Certain Central Nervous System Stimulant Drugs And Related Compounds On The In Vitro Oxygen Uptake And Enzymic Activity In The Rat Brain.

by

David Pollock

(March 1963)

This thesis is concerned with drugs which have a stimulant action on the higher functions of the central nervous system. The effects of these drugs on the electrophysiological aspects of brain functioning are described and the value of such studies in determining their principal sites of action within the brain is discussed. Their effects on the levels of certain biologically active compounds, postulated to have a transmitter function within the brain, have been reviewed briefly. The validity of theories seeking to explain the pharmacodynamic activities of such drugs on the basis of an alteration in the brain concentrations of these hypothetical transmitters has also been discussed.

Attempts to relate the pharmacodynamic activities of both central nervous system stimulants and depressants to an effect on brain energy metabolism have been discussed.

In an attempt to provide information of value in understanding the basic mechanisms of action of central stimulants, an investigation was carried out to determine the effects of a number of compounds selected from three chemical groups, namely phenylethylamines, hydrazines and iminodibenzyl derivatives and some chemically related compounds, on the *in vitro* oxygen uptake, both normal and 2,4-dinitrophenol-stimulated and on the monoamine oxidase, acetylcholinesterase and adenosinetriphosphatase activities of preparations of the rat brain. The methods used for the determination of the oxygen uptake of slices of rat brain cerebral cortex and for the estimation of the activities of these enzymes are described.

The potent central stimulants (e.g. *d*-amphetamine and amitriptyline) inhibited the respiration of cortical

slices at concentrations which were very much higher then those likely to be found in the brain following the administration of therapeutic doses of these drugs. The weaker central stimulants (e.g. phenmetrazine and isoniazid) had no effect even at very high concentrations. An exception, however, was l-amphetamine which was almost equipotent with d-amphetamine in inhibiting respiration. The central depressant, sodium barbitone, which was studied for comparison, was also inactive in this respect. Among the compounds investigated, only imipramine and the potent uncoupling agent 2,4-dinitrophenol increased the rate of respiration. In experiments using 2,4-dinitrophenol-stimulated respiration, a number of drugs, which had previously had an inhibitory effect on normal, *in vitro* oxygen uptake, produced a more marked response (e.g. pheniprazine). This observation confims the view that stimulated *in vitro* respiration is more susceptible to inhibition than normal non-stimulated respiration. It is suggested that these results indicate that it is very unlikely that the

central stimulant drugs studied produce their pharmacodynamic effects primarily by modifying the oxygen uptake.

The results of the acetylcholinesterase experiments indicate that the inhibition of this enzyme by central stimulant drugs is not an important factor in the activity of these compounds. The potent monoamine oxidase inhibitory activity of the central stimulants, iproniazid and tranylcypromine is confirmed. While, in the case of the amphetamines, the inhibition of monoamine oxidase is probably unimportant, it is suggested that this aspect of the activity of imipramine and amitriptyline may be of greater significance than at first seemed likely, since both of these compounds are considerably more active in this respect than the amphetamines.

No correlation exists between the effects of central stimulants on the adenosinetriphosphatase activity of rat brain homogenates and their pharmacodynamic activities. It cannot be concluded however, that the mechanism of action of central

stimulants is unrelated to the modification of the activity of this enzyme *in vivo*, because of the difficulty in interpreting the results of *in vitro* estimations of adenosinetriphosphatase activity which is profoundly affected even by varying the relative concentrations of cations and anions.