THE EFFECT OF CERTAIN CENTRAL NERVOUS SYSTEM STIMULANTS AND RELATED COMPOUNDS ON THE ENERGY METABOLISM IN THE RAT BRAIN.

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I N D E X

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List of Publications

Certain aspects of the work described in this thesis have been published jointly with J.J. Lewis. Copies of the following publications are to be found at the end of the thesis.

Lewis, J.J. & Van Petten, G.R. (1962). The effect of amphetamine and related compounds on the concentration of adenine nucleotides, inorganic phosphate and creatine phosphate in the rat brain. J. Pharmacol. 136, 372-377.

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Certain aspects of the work have also been described in the following communications.

Van Petten, G.R. & Lewis, J.J. The effect of amphetamine and some related compounds upon the adenosine nucleotides of rat brain. A paper read to the British Pharmacological Society Summer Meeting, Edinburgh, July 12th, 1961. (Brit. J. Pharmacol. 1, 1.).

Van Petten, G.R. & Lewis, J.J. The effects of antidepressives and some related compounds upon adenine nucleotide levels in the rat brain. A paper read to the British Pharmacological Society Summer Meeting, Oxford, July 19th, 1962.
(Brit. J. Pharmacol. 19, 1.).

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

INTRODUCTION

GENERAL

The mind of man sets man apart. Yet our knowledge of the physiology and biochemistry of the organ of the mind, the brain, remains scanty. There seems to be little doubt, however, that the extreme complexity and the many unknown factors in brain function create a fascination in the study of the central nervous system. For the pharmacologist, the study of the way in which drugs modify brain function presents no less a challenge. Indeed, drugs have been used from time immemorial to induce temporary aberrations of the mind. For example, peyote, the most active alkaloid of which is mescaline, has been used for centuries by the Kiowa Indians of North America to induce central nervous system stimulation and visual hallucinations during religious ceremonies (Osol & Farrar, 1955). It is well known that man has for centuries used coffee and tea containing caffeine, coca leaves containing cocaine, tobacco containing nicotine and beverages containing alcohol to modify his mood for social pleasure or to enhance his performance under stress.

Many of these naturally occurring drugs, notably caffeine, nicotine and alcohol, are still widely used, but as well, a large armamentarium of synthetic compounds is now at the disposal of the clinician. Although many of these synthetic compounds have found wide application for the relief of the symptoms of mental disorders, their use has extended beyond psychiatric hospitals into general medical practice where large quantities are now prescribed. Yet. for most, if not all of these drugs, the precise mechanism of action is unknown. It is not only the lack of knowledge of the mode of action of the drugs themselves, but also the fundamental lack of understanding of normal brain function and of the aetiology of mental illness, the symptoms of which certain drugs can so markedly affect, that commands research to be continued and indeed, expanded in this field of endeavour.

This thesis is concerned primarily with an attempt to find out more about the effects of three groups of central nervous stimulant drugs on some aspects of the biochemistry of the brain in the hope that such information may eventually lead to a fuller understanding of the way in which these drugs act. For clarity, it

should be pointed out that in this thesis the phrase "central nervous system stimulant drugs" is taken to include those drugs which primarily affect the functioning of the higher centres of the brain and in general produce elevation of the mood, euphoria, increased motor activity and decreased sleeping time. This group forms part of the large general group of "psychotropic drugs" which have been defined as "compounds having a pharmacodynamic effect on the higher functions of the central nervous system" (Jacobsen, 1959). The phrase "central nervous system stimulant drugs" is thus intended to exclude those drugs such as picrotoxin, leptazole, nikethamide, pentamethylenetetrazole and strychnine which markedly affect the lower parts of the central nervous system, induce convulsions and are used primarily as analeptics. As will be seen in the ensuing discussion of their development (see pages 5 - 13), synthetic central nervous system stimulant drugs fall into three main groups depending on certain differences in their chemical structure and pharmacological and biochemical effects. The first group comprises amphetamine and drugs which resemble it in their pharmacological actions

(e.g. methylphenidate, pipradrol, phenmetrazine) and are called "the amphetamine-like central nervous system stimulants". The members of the second group (e.g. iproniazid, phenelzine, tranylcypropmine, pheniprazine) are characterized by the fact that they are potent inhibitors of the enzyme monoamine oxidase in vitro and in vivo and are referred to as "the monoamine oxidase inhibitors". The drugs of the third group (e.g. imipramine, amitriptyline) are characterized both by their ability to alleviate the symptoms of certain types of depression and by their chemical similarity to phenothiazine and are called "the antidepressives resembling phenothiazine in chemical structure". This nomenclature is derived from the recent classification of psychotropic drugs described by Shepherd and Wing (1962) which was based on the suggestions of the World Health Organization Study Group (1958). A number of other classifications have been proposed (see Kletzkin, 1962 and references cited), but as Shepherd and Wing (1962) have pointed out with regard to psychotropic drugs, "the data derived from current methods of study do not delineate their actions clearly enough for anything but a provisional system

of classification". The advantages and disadvantages of the different systems of classification are not, therefore, discussed here, and the provisional nomenclature used has been set down only as an aid to further discussion in this thesis.

The Development of Synthetic Central Nervous System Stimulants.

The history of the development of synthetic drugs which stimulate the higher centres of the central nervous system has been characterized by the fact that, in many cases, they were synthesized in an attempt to find compounds with an entirely different pharmacological action or as part of more general studies on the relationship between chemical structure and pharmacological action. The appreciation of the clinical value of central nervous system stimulants often came only after considerable investigation of their other properties, and studies designed primarily to find new drugs for the treatment of mental depression are of recent origin.

Thus, the first synthetic central nervous system stimulants were discovered during the intense studies of the peripheral sympathetic nervous system

at the beginning of the present century. The observation that extracts of the adrenal medulla possessed a vasopressor action (Oliver & Schäfer, 1895) quickly led to the elucidation of the chemical structure of the active principle, adrenaline, largely through the efforts of Abel and Crawford (1897, 1899), v.Furth (1900) and Takamine (1901), and to its synthesis independently and almost simultaneously by Stolz (1904) and Dakin (1905a). The apparent discrepancy between the conclusion that the catechol nucleus was essential for adrenaline-like activity (Dakin, 1905b) and observations that amines lacking this group had an adrenaline-like action (Barger & Dale, 1910), prompted the investigation, by the latter workers, into the relationship between the chemical structure and the pharmacological action of a series of aliphatic and aromatic amines. Since these compounds were found to "simulate the effects of sympathetic nerves not only with varying intensity but with varying precision", the term "sympathomimetic" was introduced to describe their pharmacological action (Barger & Dale, 1910). Although the ability of certain sympathomimetic drugs, including ephedrine, to arouse chloral hydrate-

anaesthetized rabbits was studied as early as 1913 (Airila), a marked awareness of and interest in their central stimulant actions was not forthcoming until some twenty years later. Even then, the investigation of the vasopressor action of amphetamine (Piness, Miller & Alles, 1930) preceded by three years the first mention of its analeptic and respiratory stimulant properties (Alles, 1933). Considerable interest was, however, subsequently shown in the central stimulant properties of amphetamine and attempts were soon made to use it for the relief of the symptoms of narcolepsy (Prinzmetal & Bloomberg, 1935) and postencephalitic Parkinsonism (Solomon, Mitchell & Prinzmetal, 1937). The extensive review by Ivy and Krasno (1941) on the clinical application of the central stimulant properties of amphetamine cites many attempts to use this drug to treat various types of depression, to elevate the mood in different types of fatigue and to increase the willingness and capacity to work. Similarly, the closely related compound, methyl-amphetamine, prepared by Ogata in 1919, received little attention until 1938 when it was claimed that its central stimulant and euphoric effects could be

produced without undesirable side reactions (see Ivy & Goetzl, 1943 and references cited).

The first systematic investigation of the central stimulant properties of sympathomimetic amines in experimental animals was carried out in 1941 when a study was made of the quantitative effects of seventy-five amines on the spontaneous activity of unrestrained, un-narcotized rats (Schulte, Reif, Bacher, Lawrence & Tainter, 1941). It was reported that the phenylisopropylamines (in particular dextro-amphetamine) were particularly effective compounds with regard to minimum effective dosage, degree of stimulation, and intensity of effect per milligram of amine. In spite of the intensity and scope of this study, more than ten years were to elapse before new amphetamine-like central nervous system stimulants were to appear, and even then, they arose from studies not primarily designed to find new central nervous system stimulants.

Thus, although the compound methylphenidate was synthesized in 1944 by Panizzon during the investigation of a group of piperidine acetic acid esters, and pipradrol was synthesized in 1948 by Tilford, Shelton

and Van Campen in a search for new antihistaminic agents, their central stimulant properties were not described until 1954. Methylphenidate was then observed to produce central nervous system stimulation in experimental animals similar to, but not identical with, amphetamine (Meier, Gross & Tripod, 1954). Similarly pipradrol was reported to produce central nervous system stimulation but the effects were said to be qualitatively different from those of amphetamine (Brown & Werner, 1954). Also at this time phenmetrazine (Thomä & Wick, 1954) was synthesized and six years later phendimetrazine appeared (Stegen, Zsoter, Tom & Chappel, 1960). Although both of these compounds have been used as anorexigenic agents, they also produce considerable central nervous system stimulation (Stegen et al., 1960; Garberg & Sandberg, 1960; Knoll, 1961). Thus several new compounds with a central stimulant action similar to amphetamine, but different from it and from one another chemically, were discovered.

The period from 1952 to 1960 also witnessed the emergence of another group of central nervous system stimulant drugs whose pharmacological activity

also differed somewhat from amphetamine and its congeners. The observation that the hydrazine derivative iproniazid, initially introduced as an antitubercular drug (Fox & Gibas, 1953), also induced euphoria and stimulation in patients with tuberculosis (Selikoff, Robitzek & Ornstein, 1952), resulted in its clinical trial for the treatment of depression (Loomer, Saunders & Kline, 1957). Although first reports of the clinical effectiveness of iproniazid for the treatment of depression were enthusiastic, later judgments were more cautious (Rees & Benaim, 1959) and toxic side effects, the most serious being liver damage (Zimmerman, Rosenblum, Korn & Feldman, 1959), were reported. Nevertheless, the investigation of the wider aspects of the pharmacology of iproniazid, especially its ability to inhibit monoamine oxidase (Zeller, Barsky, Fouts, Kirchheimer & Van Orden, 1952; Zeller & Barsky, 1952), resulted in the synthesis of a large number of chemically and pharmacologically similar compounds (see Zeller, 1959, editor, symposium on amine oxidase inhibitors). Indeed, in recent years a primary criterion for the selection of drugs for further investigation for the treatment of mental depression has been their ability

to block monoamine oxidase *in vitro* and *in vivo* (Burns & Shore, 1961). Among the large number of compounds which have been shown to inhibit monoamine oxidase, phenelzine, pheniprazine, isocarboxazid, nialamide and tranylcypromine (Chessin, Dubnick, Leeson & Scott, 1959; Horita, 1958; Randall & Bagdon, 1959; Rowe, Bloom, P'an & Finger, 1959; Tedeschi, Tedeschi, Ames, Cook, Mattis & Fellows, 1959; respectively) may be cited as examples of some of the compounds of this type which have also been reported to be of use in the treatment of depression (Holt, Wright & Hecker, 1960; Crisp, Hays & Carter, 1961; Dally & Rohde, 1961).

On the other hand, certain compounds have been found which show little or no ability to inhibit monoamine oxidase, but are, nevertheless, clinically useful for the treatment of certain types of depression. From a group of tertiary-aminoalkyliminodibenzyl derivatives synthesized in 1951, which had antihistaminic, antispasmodic and local anaesthetic actions (Häfliger & Schindler, 1951; Schindler & Häfliger, 1954), the compound imipramine was later shown to have anti-depressive properties (Kuhn, 1957). Pharmacologically,

imipramine differs from the central nervous system stimulants previously mentioned because it does not tend to produce obvious signs of stimulation in normal animals (Domenjoz & Theobald, 1959). Instead, as these workers reported, it tends to resemble the tranquillizer chlorpromazine in that it exerts a taming effect on aggressive behaviour in experimental animals, reduces body temperature and potentiates hypnotics. Since imipramine only tends to produce signs of central nervous stimulation in animals sedated with a drug such as reserpine or in mentally depressed patients (Sulser, Watts & Brodie, 1962), it has been considered to represent a milestone in the development of drugs specifically for the treatment of mental disease and points out the need for new screening techniques to find such drugs. More recently, the chemically related compound amitryptyline (Freed, 1960), and the metabolite of imipramine, desmethylimipramine (Sulser et al., 1962), have also been claimed to be useful in alleviating the symptoms of certain types of depression.

Despite the rapidity with which synthetic

central nervous system stimulants have appeared in recent years, and the value of many of them in the treatment of various types of depression, their precise mechanism of action remains obscure. The now considerable research on these drugs has been concerned both with the physiological and biochemical aspects of their central action. Although the results of these two experimental approaches are no doubt related to one another, the various theories arising from them, which attempt to explain the mechanism of action of central nervous system stimulant drugs, can be conveniently considered separately.

THEORIES ON THE MECHANISM OF ACTION OF CENTRAL NERVOUS SYSTEM STIMULANT DRUGS

Physiological Aspects

Neurophysiological experiments, relying mainly upon macroelectrode techniques, have been carried out in an attempt to establish the site or sites of action of stimulant and depressant drugs within the brain. Among central stimulant drugs, considerable attention has been devoted to studies of the effect of amphetamine on the electrical activity of the brain (see reviews by Toman & Davis, 1949; Leake, 1958; Himwich, 1959).

Although a detailed review of the physiology of the central nervous system is outside the scope of this thesis, for clarity, a brief resume of the physiology of those areas of the brain believed to be implicated in the action of central stimulants will be given. Birzis (1960) considers that the closely related reticular activating system, the unspecific thalamocortical system and the limbic system may all be involved in the action of central nervous system

stimulant drugs.

Among its other important functions, the reticular activating system, consisting of the reticular formation and related thalamic nuclei, is implicated in governing the state of wakefulness (see review by French, 1960). In the sleeping or drowsy animal, high frequency stimulation (100 to 200 per second) of the midbrain reticular formation or its afferent pathways gives rise to the appearance of low amplitude fast waves in the electroencephalogram (EEG) of the cortex. This EEG arousal pattern is normally associated with behavioural signs of awakening or alertness (Birzis, 1960). For the evaluation of the effects of drugs, either the thresholds at which EEG and behavioural arousal are produced, or the duration of the post-stimulatory excitation resulting from a constant maximal stimulus, may be used as test parameters.

The unspecific thalamocortical system consists of the rostral portions of the ascending reticular activating system of the brain and a diffuse network with connections to many parts of the cortex. It is

believed to mediate and distribute, to almost all parts of the cortex, some of the activation originating in the more caudal portions of the brain stem reticular formation. It may also serve to maintain a more direct control over the rhythmic electrical activity of the cortex and may act as an intrathalamic integrating system (Jasper, 1960 and references cited). Low frequency stimulation (5 to 15 per second) of the unspecific thalamocortical system produces the recruiting response in the cortex (Morrison & Dempsey, 1942) and this EEG pattern has been compared to the spindle bursts seen in barbiturate anaesthesia and to the resting alpha rhythm of the human EEG. Although there seems to be a reciprocal relationship between the recruiting response produced by the appropriate stimulation of the unspecific thalamocortical system and the alerting response produced by the appropriate stimulation of the reticular activating system (Tissot & Monnier, 1959), it is possible to elicit either response by stimulation of a suitable locus in the rostral reticular formation where the two systems intermingle.

The limbic system is believed to consist of cortical structures such as the hippocampus, the cingulate gyrus, and entorhinal cortex, and also of subcortical masses such as the septum, amygdala, certain diencephalic nuclei, and connecting tracts (Birzis, 1960). Although the limbic system is believed to be involved in emotion and perhaps as a modulator of the other systems, neither its precise functions (see reviews by Brady, 1960; Green, 1960; Gloor, 1960) nor its anatomical definition (Brady, 1960) have been clearly established.

Attempts to elucidate the site of action of central nervous system stimulant drugs have been made by studying their effects on the electrical activity in any one or all three of these systems. Although stimulant drugs generally convert the EEG to a fast, low-voltage pattern similar to that seen in the alert behavioural state, this effect seems to be produced in different ways.

Thus amphetamine lowers the threshold for production of the EEG arousal pattern which normally results both from stimulation of the reticular formation

and certain of its afferent peripheral pathways (Konigsmark, Killam & Killam, 1958). Although the activation response produced by amphetamine is not blocked by high spinal section (Bradley & Elkes, 1957; Schallek & Kuehn, 1959), the drug is unable to activate the cortex which is cut off from the influence of the reticular formation (Elkes, Elkes & Bradley, 1954). If, however, portions of the rostral reticular formation are spared by the section, then appropriate doses of amphetamine still produce the alerting reaction. This and other experimental evidence (see reviews by Toman & Davis, 1949; Leake, 1958; Himwich, 1959; Birzis, 1960) indicate that the EEG arousal pattern produced by amphetamine is primarily due to a direct effect on the reticular activating system, possibly through an action on adrenergic neurones (Bradley & Elkes, 1957). The central stimulant drug, dimethylaminoethanol, resembles amphetamine in its ability to lower the arousal threshold to stimulation of the reticular formation, but this action is weak and inconsistent (Konigsmark et al., 1958; Himwich, 1959). In the case of dimethylaminoethanol, an action on

adrenergic neurones may not be responsible for the effect, since this drug may be a precursor of acetylcholine (Murphree, Jenney & Pfeiffer, 1959). It may, however, still exert a direct action on the reticular activating system, since both acetylcholine and adrenaline have been shown to lower the threshold of the reticular formation to stimulation (French, 1960 and references cited).

Methylphenidate resembles amphetamine in its ability to alter the EEG to an arousal type pattern but this seems to be produced by a different mechanism. Unlike amphetamine, the activation produced by methylphenidate is not abolished by lesions of the midbrain reticular formation (Jouvet & Courjon, 1959). This activation is, however, blocked by lesions of the diffuse thalamic nuclei (Jouvet & Courjon, 1959). In the case of methylphenidate, the arousal pattern may be due predominantly to excitation of rostrally-located activating neurones but may also be produced by inhibition of the recruiting response (Birzis, 1960).

Caffeine seems to produce arousal by yet another mechanism. The ability of caffeine to produce

a low-voltage activation pattern in the EEG is not abolished either by the isolation of the cortex from the reticular formation or by lesions of the diencephalic grey matter (Jouvet & Benoit, Marsallon & Courjon, 1957). Caffeine apparently inhibits the recruiting response by a direct action on the diffuse thalamocortical circuit (Krupp, Monnier & Stille, 1959). The possibility that caffeine sensitizes the cortex, by producing a relative hypoxia as a result of an increased cerebral metabolism in conjunction with a reduced blood flow, cannot, however, be discounted (Kety, 1959).

Less is known about the effects of the newer central nervous system stimulants on the neurophysiology of the brain. The following examples illustrate some of the difficulties presently associated with attempts to correlate the pharmacological action of these drugs with their effects on electrical activity in the brain. The reversible monoamine oxidase inhibitor, alpha-methyltryptamine, has been observed to produce low-voltage fast waves in the EEG similar to those produced by amphetamine (Himwich, 1961).

Although high doses (500 to 900 mg/kg) of the irreversible monoamine oxidase inhibitor, iproniazid, have been reported to produce this type of EEG pattern, the administration of 25 mg/kg daily for 5 days produced no apparently persistent change in the EEG pattern (Himwich, Costa, Pscheidt & Van Meter, 1959; Costa, Pscheidt, Van Meter & Himwich, 1960). Low doses of the related monoamine oxidase inhibitors, pheniprazine and tranylcypromine, have been reported to produce an activation type of pattern in the EEG which is slower in onset but of longer duration than that produced by amphetamine (Costa et al., 1960). More recently, however, the daily administration of iproniazid, tranylcypromine, isocarboxazid and nialamide was observed to cause a progressive day by day slowing of the EEG waves. This pattern resembled that seen in sleep, even though the animals were awake at the time the EEG was recorded (Funderburk, Finger, Drakontides & Schneider, 1962). Furthermore, the arousal threshold of the reticular activating system was not apparently modified by these four drugs. The antidepressive drug, imipramine, produces neurophysiological effects

similar to those of the phenothiazine tranquillizers. Thus it produces slowing of the EEG with high amplitude waves and spindles, and depresses the reticular activating system (Bradley & Key, 1959). On this basis there seems to be little foundation for its mood-elevating action (Birzis, 1960).

The recent suggestion that the limbic system is involved in emotional responses (MacLean, 1958) has prompted investigations of the effects of drugs which alter the mood on this part of the brain. For example, the observations that iproniazid and isocarboxazid increase the electrical activity of the amygdala, while imipramine depresses the activity of the septum, might be reconciled with the psychostimulant properties of these compounds "if the amygdala has a facilitating influence on behaviour" and "if the septum has a restraining influence on behaviour" (Schallek, Kuehn & Jew, 1962). These workers also observed, however, that the psychodepressants chlordiazepoxide and meprobamate also depress the septum. Similarly, both pentobarbitone and amphetamine reduce the duration of limbic seizures, yet these two drugs are generally

considered to produce diametrically opposite effects on the central nervous system (Kletzkin, 1962). The present difficulty in attempting to resolve such apparent discrepancies seems to be largely attributable to the general lack of knowledge of the precise function of the limbic system.

In general it seems that neurophysiological data has shown certain broad differences between centrally acting drugs such as the depressants and stimulants. Research in this field has, however, not advanced to the point of being able to associate a particular pharmacological effect with a particular change in the normal neurophysiological sequence of events in the brain. The need for more information on similarly acting drugs on neurophysiological mechanisms is apparent. By defining more specifically the sites of action of the drugs within the brain, this type of investigation would appear to offer considerable direction to investigations into the underlying biochemical means by which the electrophysiological changes are produced.

BIOCHEMICAL ASPECTS

In recent years, many attempts have been made to explain the mechanism of action of central stimulant drugs in terms of their effects on biochemical processes in the brain. For convenience, the theories put forward may be divided into those concerned with an action of these drugs on central synaptic transmission and those concerned with their effects on brain energy metabolism. This division does not, however, exclude the possibility of a relationship between synaptic transmission and energy metabolism.

Central Synaptic Transmission and the Action of Central Nervous System Stimulant Drugs.

The existence of chemical mediators, or neurohormones, to explain the transmission of impulses in the peripheral sympathetic nervous system was first clearly suggested by Elliott (1904). The subsequent application of this concept to the postganglionic parasympathetic synapses(Loewi, 1921), the neuromuscular junction (Dale, Feldberg & Vogt, 1936; Brown, Dale & Feldberg, 1936; Brown, 1937), and autonomic ganglia

(Feldberg & Gaddum, 1934) led to the view that transmission at these sites was mediated by acetylcholine. In spite of the continuing controversy concerning the precise mechanism of action of acetylcholine in peripheral neurones and at synapses (Nachmansohn, 1959; Katz, 1962; Koelle, 1962), the transmission, in higher animals, of peripheral nervous impulses from neurone to neurone or from neurone to effector cell is now generally believed to be accomplished by specific chemical substances, referred to as transmitters or neurohormones. The theory of chemical transmission at peripheral synapses has, furthermore, provided a satisfactory basis for explaining the peripheral actions of many drugs, (e.g. neuromuscular, ganglion and adrenergic blocking agents) and their antagonists.

Although the view that transmission across synapses in the central nervous system might also be mediated by chemical means was tentatively suggested in 1937 by Dale, this has not yet been unequivocally established (see review by Crossland, 1960). The nature of the evidence in its favour can be indicated

briefly. For example, changes in the membrane potential of motor neurones in the spinal cord accompanying both reflex excitation and inhibition appear to arise in the neuronal membrane itself and are not passively induced by action currents from the stimulated afferent nerves. This suggests that they are produced by chemical agents (Brock, Coombs & Eccles, 1952). Considerable support for the view that the synaptic regions of the central neurones are not excited directly by electrical means has accumulated (see review by Grundfest, 1957). Electron microscopy of the synaptic regions of the central nervous system has revealed a space of 120 to 200 Å between the pre- and post-synaptic membranes (De Robertis, 1959, 1961) and the existence of synaptic vescicles in close proximity to the presynaptic membrane (Gray & Whittaker, 1960, 1962). This appearance is consistent with the idea that the impulse is transmitted across the synapse by chemical means (Eccles & Jaeger, 1958).

The complexity of the central nervous system has, however, made the identification of the neurohormones responsible for transmission very difficult.

A considerable number of substances present in the brain have been considered as possible transmitters. These include acetylcholine (MacIntosh, 1941; Feldberg & Vogt, 1948), adrenaline and noradrenaline (Vogt, 1954), dopamine (Carlsson, 1959), 5-hydroxytryptamine (Amin, Crawford & Gaddum, 1960), histamine (Kwiatkowski, 1943), gamma-aminobutyric acid (Berl & Waelsch, 1958), substance P (Von Euler & Gaddum, 1931; Hellauer & Umrath, 1948), adenosinetriphosphate (ATP) (Holton & Holton, 1954; Holton, 1959) and an unidentified cerebellar factor (Crossland & Mitchell, 1956). Although a comprehensive review of the literature pertaining to the possible role of each of these substances in the central nervous system (see reviews by Crossland, 1960, Robson & Stacey, 1962) is outwith the scope of this thesis, a very brief attempt will be made to indicate the present situation.

The evidence is probably strongest in the case of acetylcholine. In the brain, the uneven distribution of acetylcholine (MacIntosh, 1941) is closely paralleled by the distribution of choline-acetylase necessary for its synthesis (Feldberg & Vogt, 1948; Hebb & Silver, 1956).

Cholinesterase, which is necessary for the rapid breakdown of acetylcholine, is widely distributed in the central nervous system (Burgen & Chipman, 1951). Moreover, there is histochemical evidence for the presence of acetylcholine near synaptic sites (Whittaker, 1959; Gray & Whittaker, 1962). The local application of acetylcholine, or an anticholinesterase, to the motor cortex causes an increase in the activity of the pyramidal tracts (Eccles, Eccles & Fatt, 1956). The injection of acetylcholine or anticholinesterases also causes repetitive firing of Renshaw cells, while atropine and dihydro-β-erythroidine depress their activity (Curtis & Eccles, 1958). An inverse relationship between cerebral electrical activity and acetylcholine concentration has been observed (Richter & Crossland, 1949; Crossland & Merrick, 1954). Moreover, acetylcholine can be collected from the cortex, if cholinesterase is inhibited; the amount is decreased during deep anaesthesia and none at all is obtained if the electrical activity of the cortex is brought to an end by undercutting it (MacIntosh & Oborin, 1953). More recently, the

release of acetylcholine within the cerebrum has also been demonstrated (Gaddum, 1961).

Acetylcholine may not, however, be the sole transmitter in the central nervous system. In at least some regions belonging to the same afferent and efferent pathways, there are areas of high and low choline-acetylase content. This suggests that there are alternating cholinergic and non-cholinergic neurones (Feldberg & Vogt, 1948).

There is at present little experimental evidence in favour of ATP, the cerebellar factor, substance P, or histamine acting as transmitters. There is some evidence that gamma-aminobutyric acid may act as an inhibitory substance, possibly at so-called inhibitory synapses (see review by Elliott & Jasper, 1959), but its precise function in the central nervous system remains unknown (Curtis, Phillis & Watkins, 1959). In attempts to find a central non-cholinergic transmitter, the catecholamines, adrenaline, noradrenaline and dopamine, together with 5-hydroxytryptamine have received considerable attention. The uneven distribution in the brain of these

substances (Vogt, 1954; Carlsson, 1959; Bogdanski, Weissbach & Udenfriend, 1957; Kuntzman, Shore, Bogdanski & Brodie, 1961), the close association of the enzymes required for their synthesis (Bogdanski et al., 1957; Kuntzman et al., 1961; Udenfriend & Creveling, 1959) and the wide distribution of enzymes for their rapid removal (Bogdanski et al., 1957; Axelrod, Albers & Clemente, 1959) is consistent with the view that they act as neurohormones. Histochemical evidence indicates the presence of 5-hydroxytryptamine near central synapses (Gray & Whittaker, 1960). Furthermore, drugs which increase the secretion of the adrenal medulla also cause a loss of sympathin from the hypothalamus (Vogt, 1954; Holzbauer & Vogt, 1954). Small doses of adrenaline (Bonvallet, Dell & Hiebel, 1954) and noradrenaline (Rothballer, 1956) produce an alerting reaction similar to that produced by stimulation of the ascending reticular system. Similarly, the increase in the brain level of 5-hydroxytryptamine, following injection of 5-hydroxytryptophan, is associated with signs of central stimulation (Bogdanski et al., 1958). These observations suggest

that noradrenaline and 5-hydroxytryptamine may have a neurohumoral role in the central nervous system.

The present situation can, therefore, be summarized by saying that there is strong, but not conclusive evidence, for the existence of a chemical transmitter mechanism in the brain, but neither the identity of the transmitter or transmitters nor the precise nature of the mechanism has been unequivocally established. In spite of this, considerable research has been directed towards attempts to explain the action of central nervous system stimulant drugs on the basis of their ability to modify the metabolism of hypothetical transmitters, particularly noradrenaline and 5-hydroxytryptamine, in the brain.

Thus in 1938 Gaddum and Kwiatkowski suggested that the potentiation of the cardiovascular effects of adrenaline and sympathetic stimulation produced by ephedrine might be explained if ephedrine prevented the breakdown of adrenaline. The subsequent observations that amphetamine inhibited guinea pig liver monoamine oxidase and prevented tyramine-induced depression of brain respiration <u>in vitro</u> led to the

suggestion that its central stimulant properties were due to inhibition of monoamine oxidase (Mann & Quastel, 1940). In this way, amphetamine was thought to prevent the formation of inhibitory aldehydes formed during the oxidation of normal amines in the brain. More recently, however, it has been shown that, *in vivo*, amphetamine neither protects exogenously supplied tyramine (Schayer, 1953) or 5-hydroxytryptamine (Sjoerdsma, Gillespie & Udenfriend, 1959) from oxidation nor raises brain levels of 5-hydroxytryptamine (Paasonen & Vogt, 1956). Furthermore, amphetamine has been observed to cause decreases in the brain levels of noradrenaline (McLean & McCartney, 1961; Sanan & Vogt, 1962; Baird & Lewis, unpublished) and 5-hydroxytryptamine (Paasonen & Vogt, 1956). The decrease in the ratio of noradrenaline to dopamine, observed after treatment with amphetamine *in vivo* (Baird & Lewis, unpublished) may be related to the ability of the drug to inhibit dopamine-β-oxidase *in vitro* (Goldstein & Contrera, 1961). There is also a lack of correlation between the central stimulant activity of the separate optical antipodes of

1-phenylethylamine and amphetamine and their ability to inhibit monoamine oxidase (Grana & Lilla, 1959). All of these later experiments strongly indicate that amphetamine does not owe its central stimulant activity to its rather weak ability to inhibit monoamine oxidase. The suggestion, however, that amphetamine, because of its structural similarity to adrenaline and noradrenaline, may mimic the central actions of these amines in the central adrenergic or ergotropic system (Brodie & Shore, 1957) has at present no experimental support.

The observations that a number of the newer central nervous system stimulant drugs are potent inhibitors of monoamine oxidase have, however, revived the idea that these two effects may be related. Indeed, it seems to be tacitly assumed by many that these new drugs owe their central stimulant properties in some way to their ability to inhibit monoamine oxidase *in vitro* and *in vivo*. On the other hand, many experimental results on the effects of the so-called monoamine oxidase inhibitors render the unreserved acceptance of such a view difficult.

One of the basic obstacles is that the enzyme

monoamine oxidase is capable of oxidizing a wide range of amines *in vitro*. These include tyramine, 5-hydroxytryptamine, noradrenaline, dopamine, tryptamine, adrenaline and other amines (Tabor, Tabor & Rosenthal, 1954; Weissback, Redfield & Udenfriend, 1957; Imaizumi, Omori, Unoki, Sano, Watari, Namba & Inui, 1959; Pratesi & Blaschko, 1959). It is not surprising, therefore, that inhibitors of monoamine oxidase have been found, *in vivo*, to increase brain levels of 5-hydroxytryptamine and noradrenaline (Shore, Mead, Kuntzman, Spector & Brodie, 1957), and dopamine (Holzer & Hornykiewicz, 1959). It thus becomes difficult to associate the central stimulant actions of these drugs with a change in the level of a particular amine in the brain.

Many attempts have, nevertheless, been made to show such an association. For example, the excitation produced in the rabbit by iproniazid, pheniprazine and 1-phenyl-3-hydrazinobutane was claimed to be temporally related to the rise in the level of noradrenaline in the brain rather than to the rise in the level of 5-hydroxytryptamine (Spector, Shore & Brodie, 1960). These workers also reported that these drugs did not

produce signs of central stimulation in the dog or in the cat and that in these species only the 5-hydroxytryptamine content of the brain increased. On the other hand, iproniazid, isocarboxazid, tranylcypromine and nialamide have been reported to produce central stimulation in the cat, the intensity of which was related to the degree of elevation of the level of 5-hydroxytryptamine in the brain (Funderburk et al., 1962). It has been claimed that iproniazid and tranylcypromine can cause a rise in the level of noradrenaline in the brain of the rat without there being any signs of central stimulation (Green & Sawyer, 1960). Moreover, it has been suggested that the central stimulation produced by monoamine oxidase inhibitors is more closely related to an elevation of brain levels of normetadrenaline than of either 5-hydroxytryptamine or noradrenaline (Carlsson, 1960). Other means of elevating the levels of certain amines in the brain have been no more successful in relating central stimulation to the level of a particular amine. Thus the administration of dihydroxyphenylalanine increases the brain dopamine level (Bertler &

Rosengren, 1959) and the administration of 5-hydroxytryptophan increases the brain level of 5-hydroxytryptamine (Bogdanski et al., 1958; Costa & Rinaldi, 1958). After both procedures the animals show signs of central excitation. There is, however, evidence that the same enzyme is responsible both for the conversion of dihydroxyphenylalanine to dopamine and 5-hydroxytryptophan to 5-hydroxytryptamine (Westermann, Balzer & Knell, 1958; Bertler & Rosengren, 1959; Kuntzman, Shore, Bogdanski & Brodie, 1961). Thus the effects of administering the two precursors may be non-specific, since either dopamine or 5-hydroxytryptamine may be formed in areas where they are not normally present (Costa, Gessa, Hirsch, Kuntzman & Brodie, 1962).

Certain other observations indicate that the rise in brain amine levels which follows administration of potent monoamine oxidase inhibitors may be partly due to mechanisms other than inhibition of the enzyme. For instance, 30 mg/kg of nialamide produces complete inhibition of monoamine oxidase in the brain of the cat, yet higher doses are still able to produce greater

increases in the brain levels of noradrenaline and 5-hydroxytryptamine (Funderburk et al., 1962). Similar observations in the mouse, using a number of monoamine oxidase inhibitors, for example, phenelzine, pheniprazine, β-phenylethylhydrazine and harmaline, indicate that the increase in the levels of these amines in the brain may be produced, at least in part, by some other mechanism (Dubnick, Leeson & Phillips, 1962). These observations may find some explanation from the evidence that monoamine oxidase inhibitors prevent the release of brain amines from their binding sites (Pletscher, 1956; Giarman & Schanberg, 1958; Axelrod, 1962). With regard to noradrenaline, there is now a considerable body of opinion which supports the view that O-methylation plays an important and perhaps major role in its metabolism and inactivation (Axelrod, 1960 and references cited). Thus inhibition of monoamine oxidase might not be the sole mechanism by which certain drugs increase the levels of amines in the brain. In fact, the antidepressive, imipramine, does not significantly inhibit monoamine oxidase (Pulver, Exer & Herrmann, 1960),

yet in the rat it produces a highly significant increase in the brain level of 5-hydroxytryptamine (Costa, Garattini & Valzelli, 1960).

In spite of these difficulties, Brodie and his associates have vigorously promoted a hypothesis to explain the central stimulant properties of monoamine oxidase inhibitors, which is based on their ability to increase the level of free noradrenaline in the brain (Brodie & Shore, 1957; Brodie, Spector & Shore, 1959; Costa et al., 1962). This hypothesis proposes that noradrenaline and 5-hydroxytryptamine are concerned respectively with the control of the functionally opposing ergotropic and trophotropic systems of the brain as postulated by Hess (1954). The presence of free noradrenaline at its specific receptor sites is believed to result in ergotropic predominance (increased sympathetic activity, exaggerated responses to environmental changes, enhanced motor activity). Similarly, free 5-hydroxytryptamine is believed to lead to trophotropic predominance (increased parasympathetic activity, decreased responses to environmental influences, decreased motor activity,

drowsiness and sleep). The central excitation produced by iproniazid (Brodie et al., 1959) and pheniprazine (Spector et al., 1960) has been reported to be temporally related to the rise in brain levels of noradrenaline. Furthermore, if animals are given the monoamine oxidase inhibitor, N-methyl-N-benzyl-2-propanylamine, and noradrenaline is then released from bound stores by alphamethyl-m-tyrosine, marked central stimulation is observed (Costa et al., 1962). Thus it is suggested that monoamine oxidase inhibitors owe their central stimulant properties to their ability to increase the brain level of free noradrenaline, which in turn produces ergotropic predominance (Costa et al., 1962). In view of the non-specific nature of the enzyme monoamine oxidase and the ability of inhibitors of this enzyme to increase brain levels of a number of amines other than noradrenaline, such a simplified theory cannot as yet be unreservedly accepted. Probably the most apt summary of the position at this time has been made by Sanan & Vogt (1962) who stated that "it seems impossible, at the present state of our knowledge, to decide whether monoamine

oxidase inhibitors exert their stimulant effect on the brain by raising the noradrenaline of the brain or by an action on other amines or by totally different biochemical processes".

In view of the evidence in favour of a neurohumoral role for acetylcholine in the central nervous system, modification of its central functions would seem to provide an obvious basis for explaining the central action of drugs. As yet, however, the possibility that central nervous stimulant drugs may act by altering such a mechanism has received little attention. It has been suggested that the stimulant, dimethylaminoethanol, acts as an acetylcholine precursor (Murphree et al., 1959). On the other hand, a number of central nervous stimulant drugs show anticholinergic properties. For example, diphenhydramine possesses sedative properties but the o-methyl derivative, orphenadrine, is not a sedative but produces euphoria and its anticholinergic action is twice that of diphenhydramine (Bijlsma, Harms, Funche, Terstegge & Nauta, 1955). Imipramine has also been shown to be a moderately potent anticholinergic (Domenjoz &

Theobald, 1959) and the potent anticholinergic, benactyzine, has been reported to be useful in the treatment of depression when used together with meprobamate (Alexander, 1958). Several other potent anticholinergic compounds produce powerful psychotomimetic and antidepressive effects in man (Fink, 1958, 1960; Abood, Ostfeld & Biel, 1958, 1959). Recently, in a study of the structure-activity relationships among a group of acetic acid esters of heterocyclic aminoalcohols, it was found that only compounds with an anticholinergic potency similar to atropine were effective as central nervous system stimulants (Biel, Nuhfer, Hoya, Leiser & Abood, 1962). Clinically, Ditran (a 70:30 mixture of N-ethyl-2-pyrrolidylmethyl phenylcyclopentylglycolate and N-ethyl-3-piperidyl phenylcyclopentylglycolate), which is twice as potent an anticholinergic as atropine, has been reported to be an effective antidepressive agent (Abood & Meduna, 1958). Although it has been suggested that such anticholinergic compounds may act by interfering with the action of acetylcholine in the trophotropic system, thus allowing ergotropic predominance (Biel et al., 1962),

experimental support for such a hypothesis is not available. Thus, although the action of certain central stimulant drugs has been considered to be related to their ability to alter the normal actions of acetylcholine in the brain, present-day evidence does not seem to permit a general association between these two effects.

With regard to the possibility that central nervous stimulant drugs may act by modifying the actions of other substances which have been postulated as central neurohormones, very little is known. Thus a number of compounds including amphetamine, ephedrine and caffeine did not change the brain levels of substance P (Paasonen & Vogt, 1956). Iproniazid and a number of other monoamine oxidase inhibitors have been shown to cause a rise in the brain levels of free Factor I (gamma-aminobutyric acid) (Elliott & Van Gelder, 1960) but the precise relationship of these findings to the pharmacological actions of the drugs remains obscure.

Considerable efforts have thus been made to correlate the central stimulant action of drugs with

their ability to modify the metabolism of possible brain neurohormones, in particular, noradrenaline and 5-hydroxytryptamine. From the limited examples cited it can be seen, however, that no clear-cut relationship has been demonstrated. This seems to stem largely from the lack of knowledge regarding the precise function of the possible neurohormones in the brain. It is nevertheless difficult to escape the conclusion that the advent of new central stimulant drugs has, at least in part, been responsible for stimulating interest and research into the possible functions of these substances in the brain. In parallel to endeavours to explain the central stimulant action of drugs on the basis of a modification of central synaptic transmission, a more limited attempt has been made to relate the central action of drugs to a modification of brain energy metabolism. The possibility of this "totally different biochemical process" (Sannen & Vogt, 1962) being involved in the central action of drugs is, therefore, the subject of the ensuing discussion.

Brain Energy Metabolism and Drugs Which Affect Brain Function.

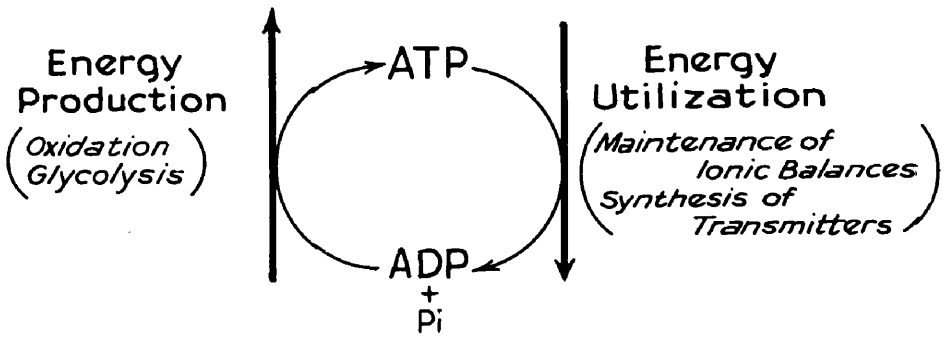
The energy required for the functioning of the central nervous system is derived almost entirely from the oxidation of glucose. A much smaller amount of energy may be derived from the glycolytic breakdown of glucose to lactic and pyruvic acids. Useful energy is obtained from both of these processes by virtue of the fact that they are coupled to the formation of adenosinetriphosphate (ATP) from adenosinediphosphate (ADP) and inorganic phosphate (Pi).

$$ADP + Pi \xrightarrow[\text{Glycolysis}]{\text{Oxidation}} ATP$$

The breakdown of the ATP formed in this way provides the energy required for the activities of the cell.

$$ATP \longrightarrow ADP + Pi + \text{Energy}$$

The relative proportions of ATP and ADP in the cell must, therefore, reflect the relative rates of energy-requiring and energy-yielding reactions as shown in the following diagram.



A net increase in energy production or a net decrease in energy utilization will be reflected by an increased level of ATP and a decreased level of ADP. Similarly, a net decrease in energy production or a net increase in energy utilization will result in a decreased level of ATP and increased level of ADP. Newly synthesized adenosinemonophosphate (AMP) may be converted to ADP by reaction with ATP

$$AMP + ATP \rightleftharpoons 2\ ADP$$

ATP can also be used to convert creatine to phosphocreatine.

$$ATP + Creatine \rightleftharpoons ADP + Phosphocreatine$$

Phosphocreatine seems to act as a readily available energy reserve which can be used for the rapid formation of ATP by reversal of the above reaction. Although a number of other phosphorylated compounds

are found in the brain, the precise functions of many of them are unknown (Heald, 1960).

The theory that drugs which affect the function of the central nervous system may do so by modifying the energy metabolism of the brain is not a new one. In particular, many attempts have been made over the past thirty years to show that drugs which depress the central nervous system do so by diminishing the energy available for its functions (see reviews by: Butler, 1950; Quastel, 1952, 1955; Brody, 1955; McIlwain, 1959, 1962; Heald, 1960; Aldridge, 1962). In 1932, Quastel and Wheatley observed that a number of narcotics including ether, chloral, nitrous oxide and several alkyl barbiturates depressed the respiration of minced guinea pig brain *in vitro*. The oxidation of glucose, pyruvate and lactate was found to be particularly sensitive to these narcotics. Moreover, brain taken from mice anaesthetized with chloroform did not oxidize glucose as well as brain from normal mice. Since the degree of inhibition of brain respiration *in vitro* appeared to parallel the narcotic potency of the compounds tested, it was suggested

that the narcosis produced by these drugs was the result of an impaired ability of the cells of the brain, or some particular area of the brain, to oxidize glucose or lactic acid and so provide the energy necessary for their normal function. It was subsequently found that the respiration of slices of brain cortex *in vitro* (Jowett & Quastel, 1937; Jowett, 1938) was depressed by lower concentrations of narcotics than those used in the early studies with minced brain. Many further studies have shown that *in vitro* brain respiration is depressed by a wide variety of central nervous system depressants, including certain steroidal anaesthetics (Gordan & Elliott, 1947), chloral (Buchel & McIlwain, 1950), chlorpromazine (Finkelstein, Spencer & Ridgeway, 1954), reserpine (Century & Horwitt, 1956), and a number of barbiturates (Fuhrman & Field, 1943; McIlwain, 1953). In many cases, however, the concentrations of these substances required to depress brain respiration *in vitro* are greater than the concentrations attained in the brain *in vivo* after therapeutic doses. Concentrations of chloral (Buchel & McIlwain, 1950),

phenobarbitone, butobarbitone and allobarbitone (McIlwain, 1953; Cohen & Heald, 1960) comparable to those which depress brain function *in vivo* produce little or no depression of brain respiration *in vitro*. Indeed phenobarbitone (Westfall, 1949), allobarbitone (McIlwain, 1953) and ethyl alcohol (Wallgren & Kulonen, 1960) in low concentrations actually produce a slight increase in cerebral respiration *in vitro*. Moreover, brain removed from rats one hour after treatment with chlorpromazine respires at a higher rate than brain removed from control animals (Century & Horwitt, 1956). The relationship between the ability of a compound to inhibit respiration of the brain *in vitro* and to produce depression of the central nervous system *in vivo* has not, therefore, been clearly established.

An alternative theory is that narcotics act not so much by inhibiting respiration itself but rather by diminishing in some way the amount of ATP which it yields (Quastel, 1952, 1955). Thus chloretone and pentobarbitone suppress the acetylation of sulphanilamide by extracts of pigeon liver when this is coupled to the production of ATP by rat brain

dispersions (Johnson & Quastel, 1953a,b). Since ATP reverses this depression and the acetylation mechanism itself is not affected by the narcotic, it has been concluded that the synthesis and not the utilization of ATP is depressed. If narcotics do decrease the yield of ATP in this way, they may do so by uncoupling oxidative phosphorylation (Brody & Bain, 1951). For example, concentrations of barbiturates such as thiopentone, pentobarbitone and amylobarbitone comparable to those found in vivo after an anaesthetic dose depress the uptake of inorganic phosphate during the oxidation of pyruvate by brain mitochondria in vitro without affecting the rate of oxidation (Brody & Bain, 1951, 1954; Bain, 1952). Under similar conditions these drugs produce the same effect as the classical uncoupling agent, dinitrophenol. Anaesthetic concentrations of ether and a steroidal anaesthetic (Truitt, Bell & Krantz, 1956) have also been shown to depress the ratio of phosphate uptake to oxygen uptake in particulate preparations of rat brain. A similar depression is obtained if cerebral mitochondria are pre-incubated with concentrations of chlorpromazine

considerably lower than those found after the administration of a therapeutic dose (Abood, 1955). Higher doses are required when no pre-incubation is carried out (Century & Horwitt, 1956; Berger, 1957). Reserpine has also been reported to decrease the ratio of phosphate uptake to oxygen uptake (Abood & Romanchek, 1957). Some well known depressants do not, however, produce this effect. Notably, ethanol, chlorbutanol, chloral, paraldehyde, urethane, morphine and anaesthetic mixtures of nitrous oxide/oxygen and xenon/oxygen do not depress the ratio of phosphate uptake to oxygen uptake in cerebral mitochondria (Levy & Featherstone, 1954; Wolpert, Truitt, Bell & Krantz, 1956). Allobarbitone and phenobarbitone (Brody & Bain, 1954; Wolpert, Truitt, Bell & Krantz, 1956) depress both oxygen and phosphorus uptake to about the same extent. Similarly, the ability of a number of barbiturates to inhibit the synthesis of glutamine in brain slices, a process known to depend on an adequate supply of ATP, is directly related to a decrease in oxygen uptake (Messer, 1958). Moreover, concentrations of chloral, butobarbitone and

allobarbitone which decrease the levels of phosphocreatine in slices of guinea pig cerebral cortex also cause a fall in oxygen uptake (Buchel & McIlwain, 1950). On the other hand, chloral at a concentration similar to that found in the brain after a sedative dose *in vivo* had no effect on either respiration or phosphorylation of cerebral cortex slices *in vitro* (Buchel & McIlwain, 1950). It appears, therefore, that an ability to depress the formation of energy-rich phosphates is not a property common to all central nervous system depressants.

It has been found that electrical stimulation of cerebral cortex slices *in vitro* causes an increase in their oxygen uptake and lactic acid production (McIlwain, 1953), a decrease in their phosphocreatine content and an increase in their inorganic phosphate level (Heald, 1954; Cohen & Heald, 1960). The metabolic rate can thus be increased to a level closer to that operating *in vivo* and partial inhibitions of reactions become more easily detectable by their overall effect (Heald, 1960). Concentrations of chloral, urethane, phenobarbitone, butobarbitone,

allobarbitone (McIlwain, 1953; Forda & McIlwain, 1953) and chlorpromazine (McIlwain & Greengard, 1957) similar to those found *in vivo* after therapeutic doses diminish markedly the increase in oxygen uptake and lactic acid production produced by electrical stimulation *in vitro*. The increased respiration of cerebral slices produced by potassium ions is also depressed by agents such as barbiturates, chloretone, ethanol and chlorpromazine in concentrations similar to those used *in vivo* (Ghosh & Quastel, 1954; Geddes & Quastel, 1956). The decrease in the phosphocreatine level and the increase in the inorganic phosphate content produced by electrical stimulation of cerebral slices *in vitro* is also inhibited by phenobarbitone in a therapeutic concentration (Cohen & Heald, 1960). Since phenobarbitone does not change the rate of increase in the phosphocreatine level immediately after stimulation ceases, it was concluded that this drug does not affect the resynthesis of phosphocreatine. The effects of narcotics on stimulated cerebral slices *in vitro* is thus consistent with the view that they depress the utilization of energy.

Experiments on oxygen uptake and phosphate metabolism *in vivo* generally support the idea that there is a diminished energy utilization in the brain during narcosis. Thus there is a decrease in the cerebral oxygen uptake during thiopentone anaesthesia in man (Kety, 1948; Wechsler, Dripps & Kety, 1951) and in the monkey (Schmidt, Kety & Pennes, 1945). Barbiturate coma and ethanol intoxication in man are also associated with a decreased cerebral oxygen uptake (Fazekas & Bessman, 1953). A decrease in both oxygen and glucose uptake by the brain in man occurs during anaesthesia induced by 21-hydroxypregnane-3,20-dione or combinations of barbiturates and pethidine (Gordan, 1956). During anaesthesia induced by a wide variety of drugs, brain levels of phosphocreatine are generally higher and ATP levels remain unchanged or are slightly increased in spite of the decreased oxygen consumption (Stone, 1940; Le Page, 1946; Lin, Cohen & Cohen, 1958; Gerlach, Doring & Fleckenstein, 1958). After treatment with chlorpromazine, an increase in the brain ATP level has been reported (Grenell, Mendelson & McElroy, 1955) but more recent studies, in which somewhat better

control values were obtained, have indicated no change in the brain level of ADP, ATP or phosphocreatine (Minard & Davis, 1962). The specific radioactivity of ATP in the brains of mice following the intracisternal injection of radioactive phosphate is, moreover, greater in animals anaesthetized with thiopentone or amylobarbitone than in control animals (Bain, 1957). This also suggests that the synthesis of ATP is not impaired. On the other hand, subnarcotic doses of phenobarbitone (Heim & Estler, 1961) and of reserpine (Kirpekar & Lewis, 1959) and related depressants (Kaul & Lewis, unpublished) decrease the brain levels of ATP. In these cases it is possible that there is a decreased production of ATP.

In summary, the concentrations of depressants required to inhibit brain respiration *in vitro* are generally higher than those found in the brain *in vivo* after a therapeutic dose. Some, but not all, narcotics appear to uncouple oxidative phosphorylation as judged by the *in vitro* depression of the ratio of phosphate uptake to oxygen uptake. In concentrations similar to those found *in vivo*, certain depressants inhibit

the increase in oxygen uptake and the decrease in phosphocreatine level produced in slices of cerebral cortex by electrical stimulation. Similarly, *in vivo*, anaesthesia produced by a number of drugs is accompanied by a decrease in the oxygen uptake of the brain, yet there is no change or an increase in the brain levels of energy-rich phosphates. This is consistent with the view that there is a decrease in the utilization of energy by the brain during narcosis induced by many drugs. The suggestion that narcotics depress energy production in a particular area of the brain and that this in turn results in a general depression of the activity of the rest of the brain (Quastel & Wheatley, 1932; Quastel, 1952) lacks experimental support. Further experimentation on the effects of mild sedatives on brain energy metabolism are required to substantiate the possibility that their action may be associated with a depression of energy production in the brain.

Much less is known about the effects of central nervous system stimulants on brain energy metabolism. Little or no indication is given by *in vitro* studies that these drugs may act by modifying energy metabolism

in the brain. Thus amphetamine produces no effect on the respiration of brain slices *in vitro* unless high concentrations are used (Mann & Quastel, 1940; Lu & Krantz, 1953). While caffeine has been observed to increase the oxygen consumption of slices of rat brain cortex, ephedrine and amphetamine produce inhibition (Levy, 1946). Using a similar preparation, Weiner (1959) reports that amphetamine, mephenteramine and iproniazid produce no consistent effects. High concentrations (10^{-2} to 10^{-4} M) of amphetamine, methylamphetamine, ephedrine, phenmetrazine and iproniazid have been shown to depress the respiration of slices of rat brain cortex metabolizing glucose (Lewis & Pollock, unpublished). Under similar conditions, low concentrations (10^{-5} M) of the anti-depressive drugs, imipramine and amitryptyline, markedly depress oxygen uptake.

Similarly, central nervous system stimulants seem to produce no consistent effects on the respiration of the brain *in vivo*. Amphetamine may slightly decrease (Abreu, Liddle, Burks, Sutherland, Elliott, Simon & Margolis, 1949) or cause no change in cerebral metabolism *in vivo*

(Kety, 1959). Caffeine produces no effect (Shenkin, 1951) or a slight increase (Kety, 1959) in the oxygen uptake of the brain in man and no effect on either cerebral circulation or metabolism occurs *in vivo* after the administration of methylphenidate (Ehrmantraut, Shea, Ticktin & Fazekas, 1957). Thus neither *in vitro* nor *in vivo* studies of the effects of central nervous system stimulants on the respiration of the brain indicate that these drugs may act by modifying energy metabolism.

On the other hand, certain *in vivo* studies suggest that central nervous system stimulants modify the phosphate metabolism of the brain. Thus Palladin (1952) found that four hours after treatment with methylamphetamine, the ATP content of the rabbit brain was doubled (Palladin, Khaikina & Polyakova, 1952). On this basis it was suggested that the central stimulation produced by methylamphetamine was due to an increase in the energy available for brain functions. The brain level of ATP has also been observed to increase when central nervous system stimulation is produced in the rat by caffeine (Mison-Crighel,

Constantinescu & Crighel, 1959) and in the mouse by both 3-3-iminodiproprionitrile (Harth & Mandel, 1958) and ether (Estler & Heim, 1960). The observation that the rate of incorporation of radioactive phosphate into ATP in the brain is greater after treatment with methylamphetamine indicates that the metabolic exchange of ATP is increased after this central nervous system stimulant is given (Palladin & Rybina, 1953). Observations such as these are probably most interesting when they are compared to the effects of "physiological stimulation" of the brain. In this case, if emotional excitement is produced in an experimental animal by rotating it in a drum, applying electric shocks or simply by teasing it with a straw, the ATP content of the brain falls (Le Page, 1946; Sytinsky, 1956; Shapot, 1957). This is not surprising since under such conditions the brain may require a greater amount of energy to support its increased activity. The increase in the ATP level of the brain observed during central stimulation produced by drugs, when presumably the brain is again requiring more energy for its functions, may therefore be due to some direct

effect of the drug on brain energy metabolism. Not all investigators have, however, found an increase in the content of ATP in the brain after treatment with central nervous system stimulants. Thus Palladin and Rybina (1953) report that one hour after treatment with methylamphetamine, the brain ATP content is decreased. It then rises slowly, reaching a normal level within two hours, and is above normal four hours after treatment. A decrease in the content of ATP in the brain has also been found after treatment with pheniprazine (Bernsohn, Possley & Custod, 1959). At the present time it is, therefore, difficult to relate the central stimulation produced by drugs to their ability to increase the ATP level of the brain.

STATEMENT OF THE PROBLEM

The preceding survey of the literature indicates that two main theories have arisen in an attempt to explain the mechanism of action of central nervous system stimulants. The theory that these drugs modify synaptic transmission in the central nervous system is hampered by a lack of knowledge of the precise mechanism of central synaptic transmission. On the other hand, there is very little experimental evidence to support the theory that central nervous system stimulants owe their action to an ability to increase the energy available for brain functions. The present investigation was, therefore, undertaken in an attempt to find out whether or not a relationship does in fact exist between the central stimulant action of certain central nervous system stimulant drugs and their ability to increase brain ATP levels in the rat. In order to try to obtain a more complete picture of the effects of such drugs on energy metabolism in the brain, the levels of AMP, ADP, inorganic phosphate and phosphocreatine were also determined. Since anaesthesia may affect the levels of ATP and

phosphocreatine in the brain, the use of anaesthetic agents, in conjunction with the central nervous system stimulants being studied, was precluded. The effects of a number of amphetamine-like central nervous system stimulants, monoamine oxidase inhibitors, antidepressives resembling phenothiazine in chemical structure, and certain related compounds were studied. An attempt was made to find out if the potency of the different members of each of these groups of drugs as central nervous system stimulants was related to their ability to change the levels of adenine nucleotides, inorganic phosphate and phosphocreatine in the brain. A further attempt was made to determine whether the effect of a number of the drugs on the phosphate metabolism of the brain was qualitatively similar at different times during which behavioural signs of central nervous system stimulation could be observed.

M E T H O D S

Notes:

1. The methods of preparation of the reagents and the quality of the chemicals used are shown in Appendix I.

2. During the latter part of this work deionized water (conductivity less than 1.5×10^{-5} ohms/cm; "Elgastat type B.102" deionizer) was used in place of distilled or triple distilled water.

The methods used in the experiments described in the introduction were developed in the following order.

1. Methods for estimating adenine nucleotides, inorganic orthophosphate and phosphocreatine.
2. Methods for killing the rats, removal of the brains and extracting the acid-soluble phosphates.
3. Standard procedures for preparing solutions of the drugs to be studied.

Finally, an experimental design was devised to facilitate statistical analysis of the results.

ASSAY OF ADENINE NUCLEOTIDES

The necessity of assaying large numbers of tissue extracts precluded the use of the time consuming salt precipitation, chromatographic, and electrophoretic techniques for the separation and assay of adenine nucleotides (Heald, 1960 and references cited therein). Attention was, therefore, focussed on enzymic methods. Two of these appeared applicable to the present study.

The first method makes use of the fact that nicotinamide adenine nucleotide (NAD) has an absorption

maximum at a wavelength of 340 mμ in the reduced form but not in the oxidized form. By means of a series of enzyme-catalyzed reactions with appropriate substrates, the conversion of AMP to ADP, ADP to ATP, or ATP to ADP can be coupled stoichiometrically to the conversion of NAD from the reduced to the oxidized form. The resulting decrease in optical density at 340 mμ can be used to calculate the amount of AMP, ADP or ATP originally present (Kratzing & Narayanaswami, 1953; adapted from Slater, 1953; Boehringer).

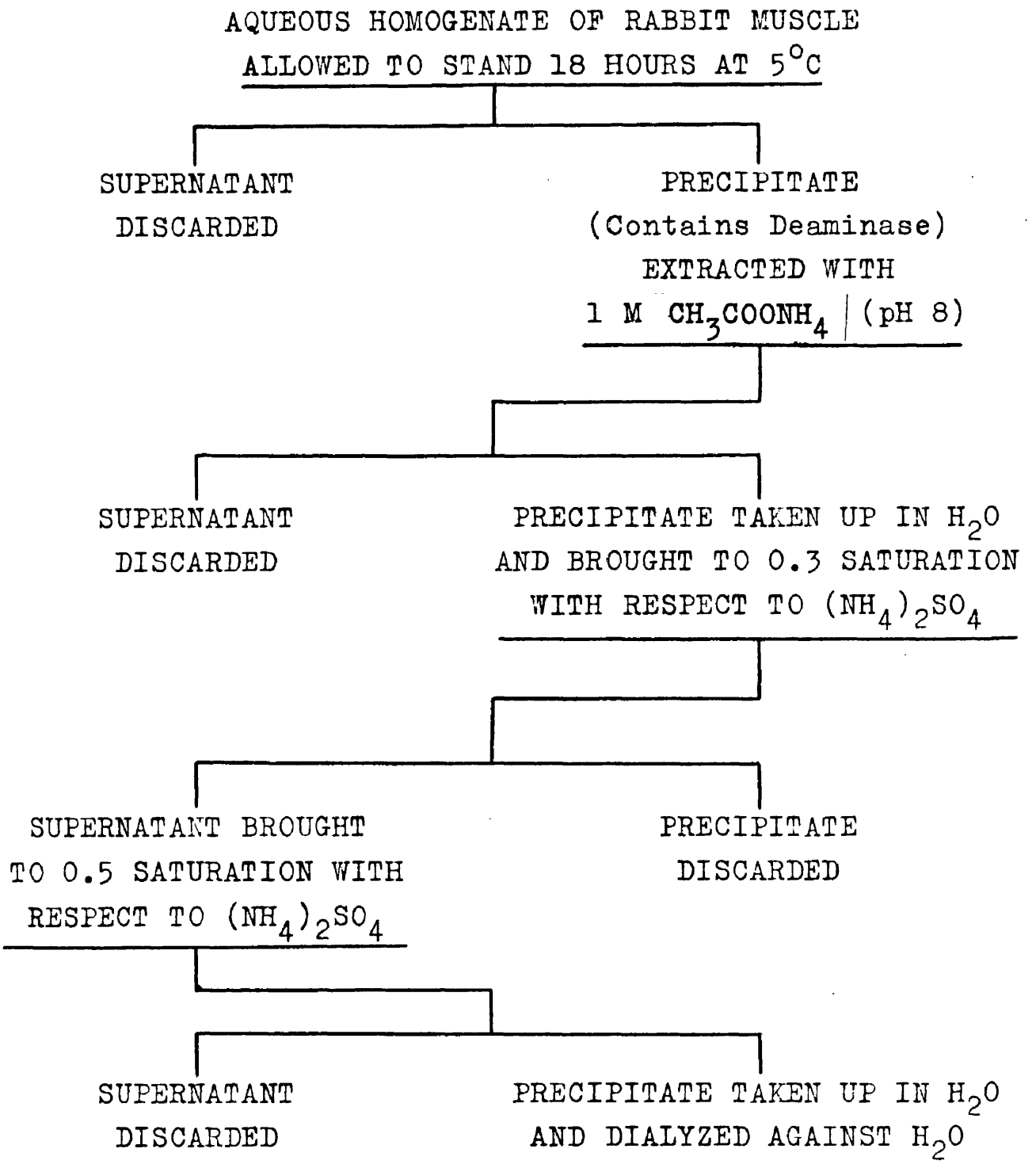
The second method is based on the fact that AMP, ADP and ATP all have an absorption maximum at 265 mμ, whereas the absorption maximum of inosine monophosphate (IMP) is at 240 mμ (Kalckar, 1947a,b,c). Conversion of AMP to IMP, using deaminase, results in a decrease in the optical density at 265 mμ which is proportional to the amount of AMP originally present. Moreover, two molecules of ADP can be converted to one molecule of AMP and one molecule of ATP by myokinase. In the presence of deaminase, the deamination of the AMP so formed results in a further fall in optical density which is proportional to one-half of the amount of ADP originally

present. ATP can be converted to AMP by apyrase and again, if deaminase is present, the optical density decreases in proportion to the amount of ATP originally present. Thus the concentrations of AMP, ADP and ATP can be determined in one run in the spectrophotometer by recording the decrease in optical density after the successive addition of deaminase, myokinase, and apyrase respectively.

Each of these methods has its advantages and disadvantages. The first method can be carried out with commercially available reagents (although these are expensive) but the actual determination is relatively complicated and less sensitive. The second method was adopted mainly because of its greater sensitivity and because AMP, ADP and ATP can be determined in a single run in the spectrophotometer. A commercial preparation of myokinase was used (obtained from C.F. Boehringer and Soehne GmbH, Mannheim, Western Germany). The deaminase and apyrase were prepared as described below.

Figure 1

PREPARATION OF DEAMINASE
(Flow Sheet)



Preparation of Deaminase (Figure 1).

Rabbit muscle deaminase was partially purified using a modification of "method B" described by Kalckar (1947c). A rabbit (3-4 kg) was killed by the intravenous administration of sodium pentobarbitone (60 mg/kg) and approximately 75 to 100 g of skeletal muscle quickly removed to a petri dish embedded in crushed ice. Using an "M.S.E. micro homogenizer", with the 100 ml vortex beaker surrounded by crushed ice, 50 g of chilled muscle was homogenized in two batches (14,000 r.p.m. for 3 minutes each) with a total of 200 ml of ice-cold distilled water. The homogenate was then kept at 4 °C for 18 hours, during which time the lactic acid, formed from the muscle glycogen, acidified the mixture to pH 6, thus precipitating the deaminase. The precipitate was removed by centrifugation (0°C, 30 minutes, 2200 x g). The deaminase was extracted from the precipitate by washing it twice with 150 ml of ice-cold 1 M ammonium acetate (pH 8). In the course of these extractions, the precipitate became gelatinous and difficult to centrifuge off using the above conditions (i.e. 0°C, 30 minutes, 2200 x g). The procedure was, therefore, modified, the precipitate being extracted

only once with 150 ml of ice-cold ammonium acetate and the centrifugation carried out in a Spinco centrifuge (0°C, 60 minutes, 78,000 x g). This modification markedly improved the yield of enzyme. The supernatant was then warmed slowly to 20°C and fractionated with ammonium sulphate. In the early preparations of deaminase, the fractionation was done by adding the required amounts of powdered ammonium sulphate to the supernatant with the aid of mechanical stirring. The low activity of the enzyme prepared in this way prompted the use of a saturated solution of ammonium sulphate to effect the fractionation while minimizing the danger of denaturing the enzyme by localized high salt concentrations (Figure 2). Thus by slowly adding 3/7 of the volume of the supernatant of saturated ammonium sulphate solution, the supernatant was brought to 0.3 saturation, with respect to ammonium sulphate. The precipitate so formed was removed by centrifugation (20°C, 30 minutes, 2200 x g) and discarded. The solution decanted off was brought to 0.5 saturation, with respect to ammonium sulphate, by making it up to twice the original volume of supernatant with saturated

ammonium sulphate solution. The precipitate so formed contained the highest deaminase activity. It was removed as before, taken up in 15 ml of ice-cold distilled water and dialyzed overnight against cold distilled water at 4°C.

The activity of the enzyme was then tested using the following method. To 1 ml of AMP (5 µmoles) was added 16.7 ml of ice-cold 0.3 M perchloric acid (PCA) and the volume adjusted to 100 ml with ice-cold triple distilled water. To 10 ml of this solution was added 10 ml of 0.16 M succinate buffer (pH 6.1), 1 ml of 0.1 M $CaCl_2$ and 1 ml of 1 M $MgCl_2$. The pH was adjusted to 6.1 by titration with 1 N NaOH, using a glass electrode assembly, and the volume adjusted to 25 ml with triple distilled water. A reagent blank containing no AMP was similarly prepared. The optical density of a 3 ml aliquot of the reagent blank and of duplicate 3 ml aliquots of the buffered AMP solution were read against triple distilled water at a wavelength of 265 mµ using a spectrophotometer. Following two consecutive identical readings of the optical density of these three solutions, 0.02 ml of the enzyme was

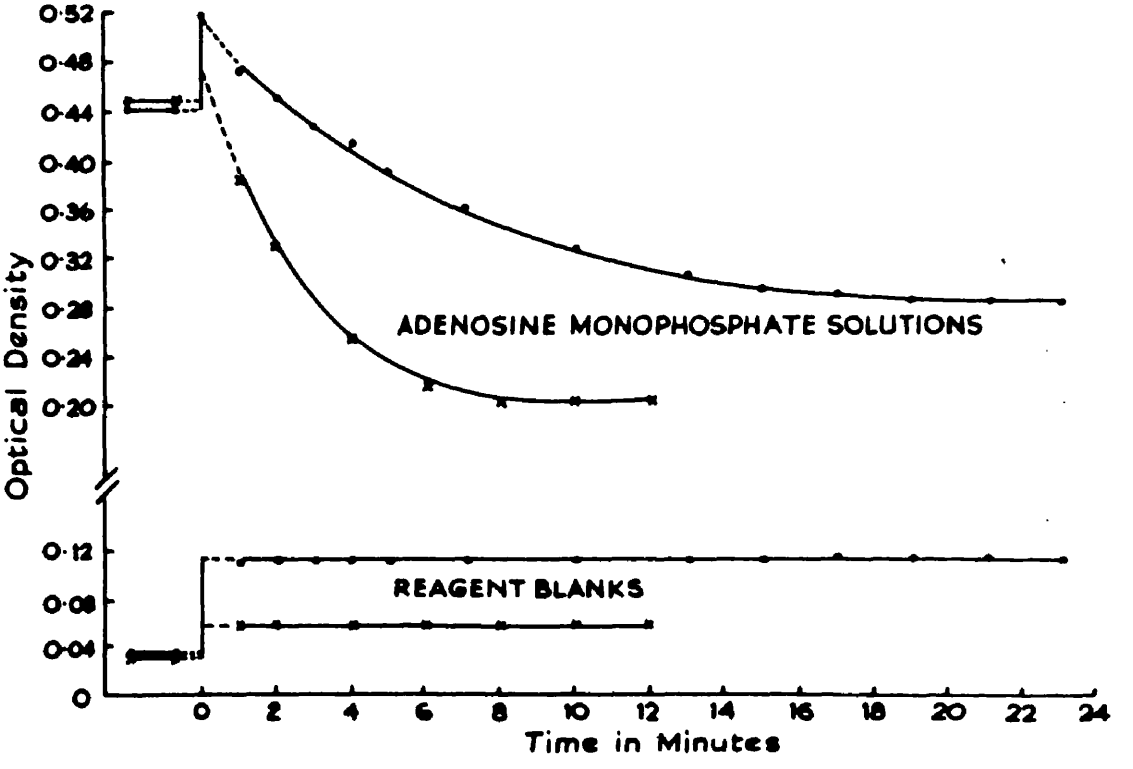


Figure 2

The Activity of Deaminase Prepared by Different Methods.

The graph shows the change in optical density observed when 0.02 ml of deaminase was added to the solutions.

o———o Curve obtained on addition of deaminase prepared using powdered ammonium sulphate for the fractionation.

x———x Curve obtained on addition of deaminase prepared using a saturated solution of ammonium sulphate for the fractionation.

Note the higher rate of reaction when deaminase prepared using a saturated solution of ammonium sulphate was used.

added to each, using an automatic constriction pipette, and the solutions briefly stirred. The optical density was then determined at 2 minute intervals until no further decrease between two consecutive readings for the buffered AMP solutions was found. In order to correct for absorption of the enzyme, the rise in optical density observed after addition of the enzyme to the reagent blank was added to the observed optical density of the buffered AMP solutions before the enzyme was added to these (Figure 2). The fall in optical density due to the conversion of AMP to IMP was, therefore, calculated by taking the difference between the corrected zero time optical density of the buffered AMP solution and its optical density when this remained constant (Figure 2; Figure 4b). Replacement of the AMP by ADP or ATP in this procedure showed that there was no appreciable fall in optical density after addition of deaminase to these two substrates. The deaminase was, therefore, considered to be sufficiently specific to be used in the combined assay of AMP, ADP, and ATP. Estimations of different concentrations of AMP using this method indicated that the same calibration factor

Table 1

The Estimation of Standard Solutions of Adenine Nucleotides.

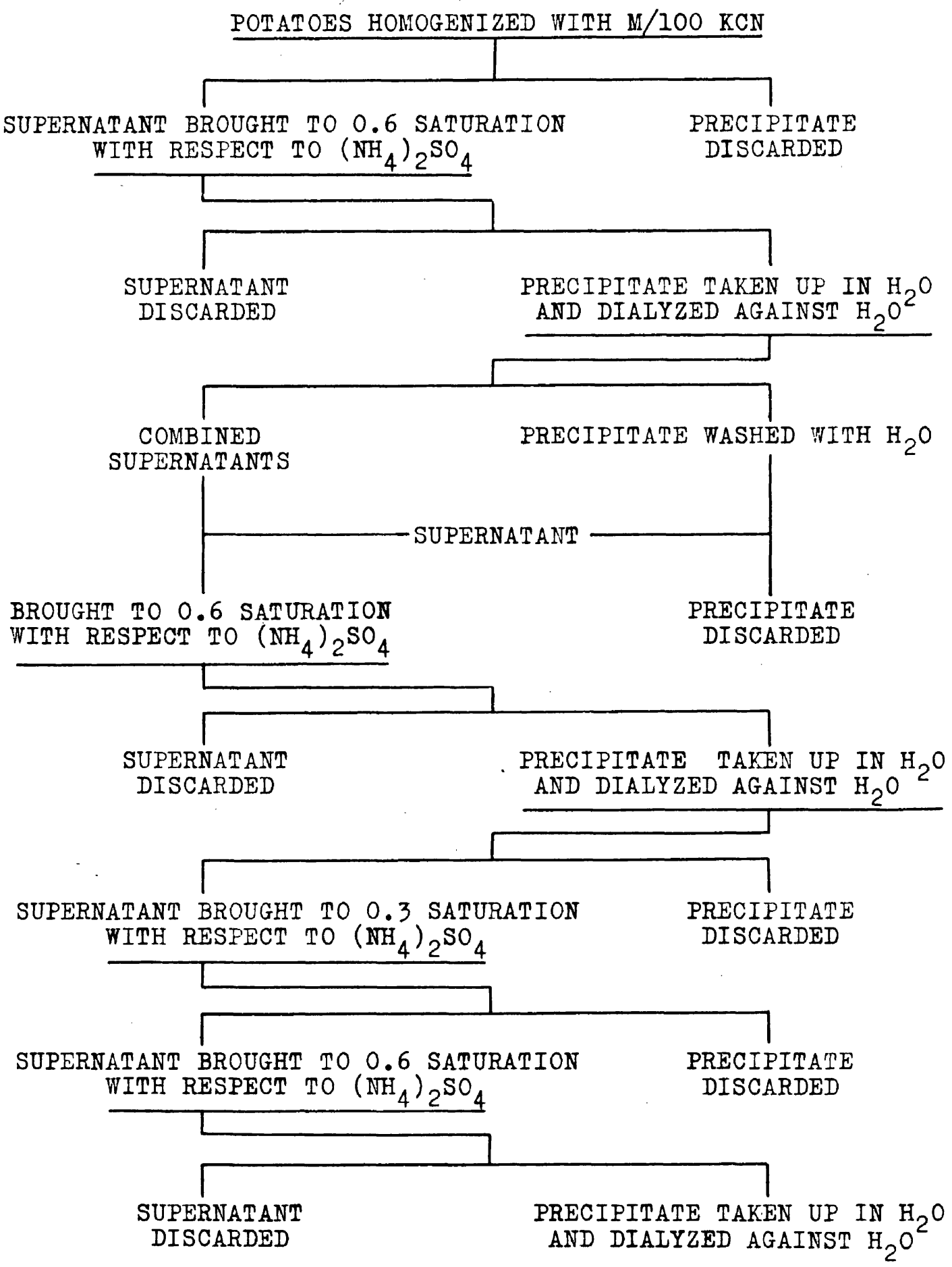
All concentrations are those of the buffered solution in the spectrophotometer cell. The theoretical concentrations are based on a weighed amount of each adenine nucleotide.

Number of Estimations	Theoretical Concentration (mμmoles/ml)	Mean Concentration Found Using Factor of 7.34. (mμmoles/ml ± S.D.)
ADENOSINEMONOPHOSPHATE		
8	9.68	9.72 ± 0.53
8	16.1	16.5 ± 1.2
10	32.6	32.7 ± 1.7
ADENOSINEDIPHOSPHATE		
2	8.0	8.04 ± 0.55
4	9.68	9.75 ± 0.97
4	16.1	15.6 ± 1.5
3	32.6	31.2 ± 0.4
ADENOSINETRIPHOSPHATE		
2	6.18	6.19 ± 0.29
12	20.0	20.0 ± 0.81
4	25.3	25.7 ± 2.0

used by Furchgott and De Gubareff (1958) was applicable to the present investigation (Table 1). In view of the importance of deaminase as the rate limiting factor in the assay of all three adenine nucleotides by Kalckar's (1947a,b,c) method and the possibility of errors occurring due to breakdown of ADP and ATP during prolonged runs in the spectrophotometer, no deaminase preparation was used which did not complete the deamination of 5 µmoles of AMP, under the above conditions, within 10 minutes. Furthermore, the enzyme was sufficiently active to enable it, when diluted three-or four-fold, to complete the above reaction in 10 to 15 minutes. The lower blank absorption of such an enzyme appeared to contribute considerably towards lowering the variation between duplicates. The enzyme was stored in the concentrated form in 1 ml aliquots in tightly stoppered vials either in the liquid form at 4°C or in the frozen state at -15°C. Large amounts of highly active deaminase were stored at -15°C to prevent the development of bacterial growths. After 2 to 3 months, however, flocculation was observed. Nevertheless, an adequately active enzyme solution

Figure 3

PREPARATION OF APYRASE
(Flow Sheet)



could be prepared by extracting the frozen aliquot with one-half the volume of 1.2 M ammonium sulphate solution usually used to dilute it and removing the precipitate by centrifugation (0°C, 10 minutes, 2200 x g).

Preparation of Apyrase (Figure 3).

Apyrase (potato adenylpyrophosphatase) was prepared using a modification of "method 1" described by Krishnan (1949). New potatoes were washed, peeled, and 500 g cut into pieces and ground for 3 minutes in a Waring Blendor with 500 ml of ice-cold M/100 potassium cyanide solution. The foam was skimmed off and the cell debris removed by centrifugation (0°C, 30 minutes, 1000 x g). Approximately 550 ml of clear, yellow-coloured, supernatant were decanted off and finely powdered ammonium sulphate (45 g/100 ml) was slowly added, with the aid of mechanical stirring, to bring the solution to 0.6 saturation with respect to this salt and thus precipitate the apyrase. Centrifugation (0°C, 45 minutes, 1000 x g), rather than overnight filtration (Krishnan, 1949), was used to remove the precipitated enzyme. The precipitate

was taken up in 60 ml of ice-cold distilled water and dialyzed at 5°C against a slow continuous flow of cold distilled water. The dialysis was further facilitated by mechanically rotating the dialysis sac slowly in the distilled water. After the passage of about 50 litres of distilled water through the apparatus over a period of about 4 hours, the fluid surrounding the dialysis sac usually gave no precipitate with acidic barium chloride solution. To complete the dialysis, the sac was placed in 4 litres of cold distilled water and allowed to stand overnight at 5°C.

During the dialysis a brown precipitate of insoluble apyrase (Krishnan, 1949) formed in the dialysis sac. The material from the dialysis sac was, therefore, centrifuged, the supernatant removed to the refrigerator, and the precipitate washed successively with 30 ml and 20 ml of ice-cold distilled water. All the supernatants were combined and all the separations carried out by centrifugation (0°C, 20 minutes, 2200 x g). The combined supernatant was warmed to 20°C and brought to 0.6 saturation with respect to ammonium sulphate as before. The resulting precipitate

was removed by centrifugation (20°C, 30 minutes, 2200 x g), taken up in 30 ml of ice-cold distilled water and dialyzed as before. On this occasion, much less distilled water was required to remove the ammonium sulphate than after the first 0.6 saturation. The small amount of insoluble apyrase formed during this dialysis was removed by centrifugation (0°C, 30 minutes, 2200 x g). The supernatant was warmed to 20°C and brought to 0.4 saturation by the slow addition of finely powdered ammonium sulphate (30.2 g/100 ml). The precipitate was removed by centrifugation (20°C, 30 minutes, 2200 x g) and the supernatant brought to 0.6 saturation by the slow addition of finely powdered ammonium sulphate (15.1 g/100 ml). The small white precipitate, which contained the purest fraction of apyrase, was then removed by centrifugation (20°C, 30 minutes, 2200 x g), taken up in 8 to 10 ml of ice-cold triple distilled water, and dialyzed against cold distilled water overnight at 5°C.

The pale yellow-coloured solution was removed from the dialysis sac and its activity estimated by substituting ATP for AMP in the method described for

testing deaminase. Deaminase was first added to the reagent blank and the buffered ATP solutions in the spectrophotometer cells and upon the completion of any small decrease in optical density, due to the presence of small amounts of AMP, 0.02 ml of apyrase was added. The optical density was read at 2 minute intervals until no further decrease was observed. Apyrase prepared by this method was found to be free from AMP-phosphatase activity which would result in low ATP values (Furchgott & De Gubareff, 1958).

During the first part of this investigation the apyrase was stored in 2 ml aliquots in small opaque vials at 5°C. In later work, however, a larger amount of apyrase was prepared by doubling all quantities in the procedure described. In this case, bacterial growths developed in the apyrase before each batch was completely used. To overcome this, the enzyme was stored in the frozen state at -15°C.

Estimation of AMP, ADP and ATP in Brain Extracts.

This was done in a manner similar to that described for the estimation of AMP by replacing the

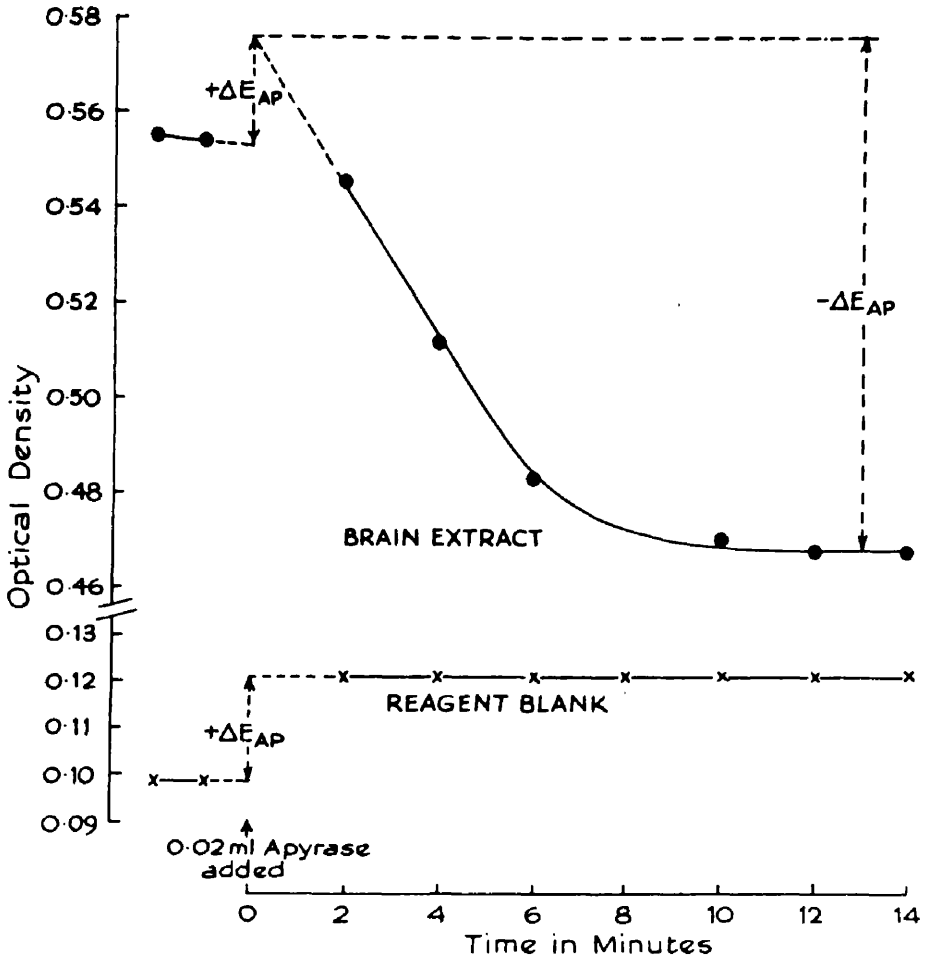


Figure 4

(c) Changes in Optical Density at 265 mμ During Analysis of Adenosinetriphosphate (ATP) in a Brain Extract.

$+\Delta E_{AP}$ represents the increase in optical density obtained in the reagent blank on addition of apyrase. In this example, $+\Delta E_{AP} = 0.022$.

$-\Delta E_{AP}$ represents the fall in optical density due to the action of apyrase on ATP and subsequent action of deaminase on the AMP formed. In this example $-\Delta E_{AP} = 0.109$. $-\Delta E_{MK}$ (Figure 4b) must be subtracted from this to correct for the ATP formed by the action of myokinase. Thus $\frac{0.109 - 0.015}{7.34}$ or 0.0128 μmoles/ml of ATP was originally present in the spectrophotometer cell.

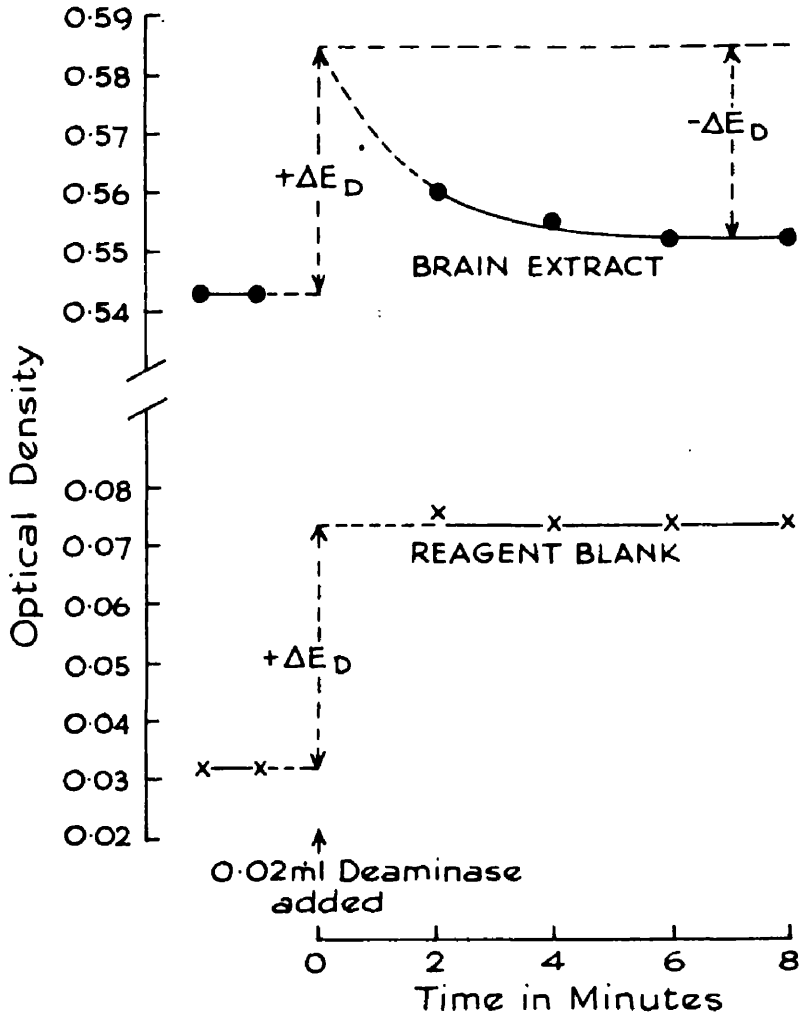


Figure 4

(a) Changes in Optical Density at 265 mμ During Analysis of Adenosinemonophosphate (AMP) in a Brain Extract.

$+\Delta E_D$ represents the increase in optical density obtained in the reagent blank on addition of deaminase and is due to the ultraviolet absorption of the enzyme preparation. In this example, $+\Delta E_D = 0.042$.

$-\Delta E_D$ represents the fall in optical density due to the action of deaminase on AMP. In this example, $-\Delta E_D = 0.033$ which is equivalent to $\frac{0.033}{7.34}$ or 0.00449 μmoles/ml of AMP in the solution in the spectrophotometer cell.

10 ml of AMP solution with 10 ml of a PCA extract of the brain. The decreases in optical density observed after the successive addition of 0.02 ml of deaminase, myokinase and apyrase to the buffered brain extract in the spectrophotometer cells were used to calculate the concentration of AMP, ADP and ATP respectively. Following the addition of myokinase, there was a slow non-specific fall in optical density and the manufacturer's instructions for extrapolating these optical density readings to the zero time of addition of this enzyme were strictly adhered to. If this is not done, falsely high ADP values are obtained. An example of the estimation of AMP, ADP, and ATP in a brain extract, together with the method of calculation, is illustrated in Figure 4a,b,c, and d.

ESTIMATION OF INORGANIC PHOSPHATE AND PHOSPHOCREATINE

The common methods for the estimation of inorganic orthophosphate make use of the blue colour developed upon the production of reduced phosphomolybdate (Fiske & Subbarow, 1925; Allen, 1940). This reaction can be used for the specific determination of inorganic

phosphate in tissue extracts provided that the reagents used and the conditions of the reaction are such that there is no hydrolysis of phosphate esters (Lowry & Lopez, 1946; Peel, Fox & Elsden, 1955; Bruemmer & O'Dell, 1956; Furchgott & De Gubareff, 1956). Phosphocreatine can be estimated in such extracts by making use of the fact that its phosphate group is much more easily removed by treatment with acid than are the phosphate groups of the other esters normally present (Lowry & Lopez, 1946; Furchgott & De Gubareff, 1956; Heald, 1960 and references cited therein).

For this investigation, a modification of the sensitive microprocedure, described by Furchgott and De Gubareff (1956), for the combined estimation of inorganic phosphate and phosphocreatine in one reaction mixture was used. In this method, inorganic phosphate is first rapidly determined colorimetrically under weakly acid conditions (pH 2.3). Since there is a very slow hydrolysis of phosphocreatine during this stage of the reaction, copper ions are added to accelerate the formation of the phosphomolybdate from the inorganic phosphate present so that this is

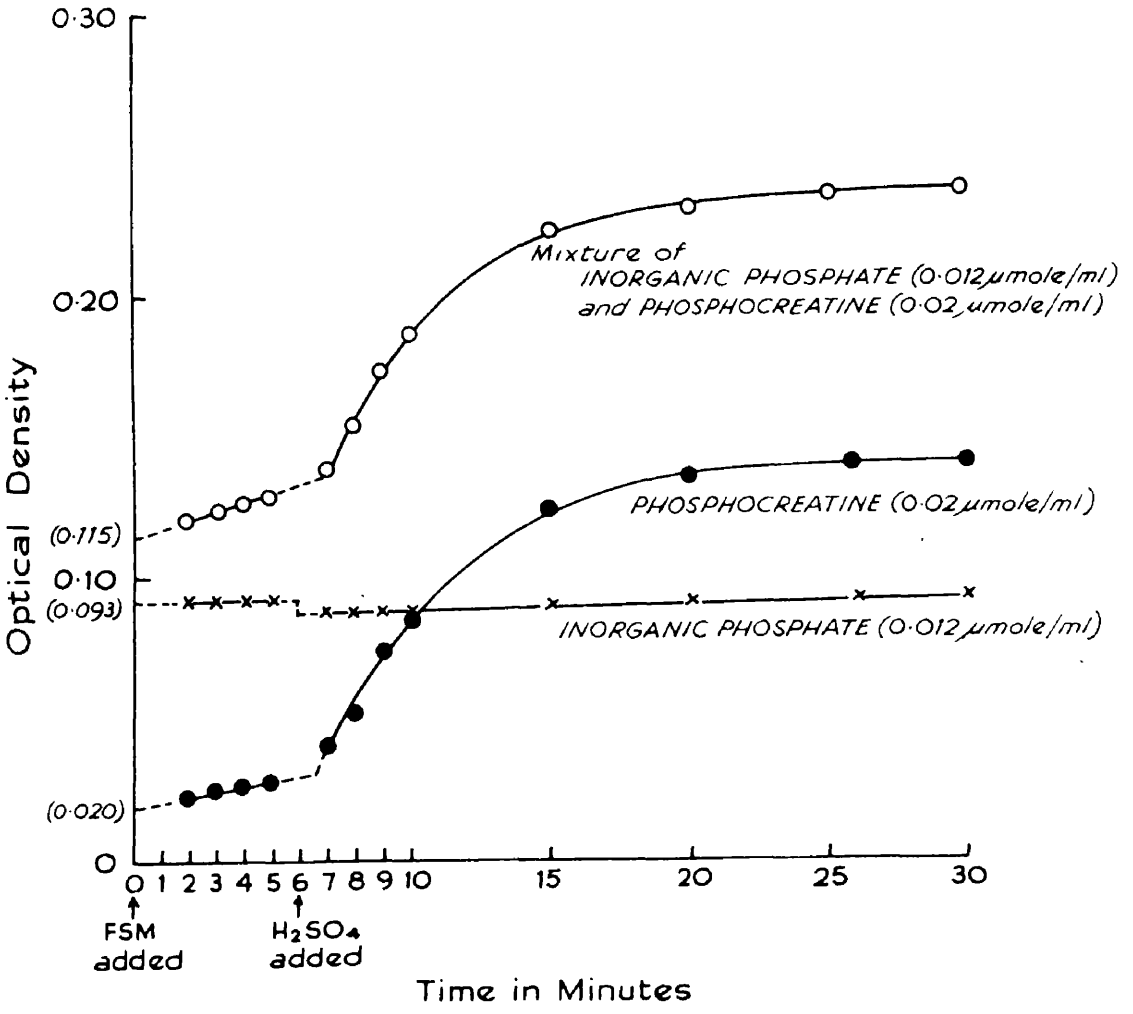


Figure 5

Determination of Inorganic Phosphate in the Presence of Phosphocreatine.

The amount of inorganic phosphate (Pi) is determined by extrapolating the 2 to 5 minute optical density readings to zero time. Thus,

Pi of solution of inorganic phosphate and phosphocreatine $\equiv 0.115$

Pi of solution of phosphocreatine $\equiv 0.020$

Pi of solution of inorganic phosphate $\equiv 0.093$

Since $0.115 - 0.020 \doteq 0.093$, inorganic phosphate can be determined in the presence of phosphocreatine.

The addition of H_2SO_4 hydrolyzes the phosphocreatine.

completed within 2 minutes of starting the reaction. Under these conditions any further colour developed after this time is due to the hydrolysis of phosphocreatine. Thus, by measuring the optical density at 1 minute intervals for 5 minutes and extrapolating the 2 to 5 minute readings to the zero time of starting the reaction, it is possible to determine the colour developed only from the inorganic phosphate present (Figure 5). Sulphuric acid is then added to the mixture in the colorimeter cell and the extra colour formed, which is due to the ensuing rapid hydrolysis of phosphocreatine, is measured. Under the conditions of the experiment, this is complete in 30 minutes. The difference in optical density at 30 minutes and that at zero time can, therefore, be used as a measure of the phosphocreatine concentration (Figure 6). Thus both inorganic phosphate and phosphocreatine are determined in one continuous run in either a colorimeter or a spectrophotometer.

The larger amounts of tissue available for assay, compared to those used by Furchgott and

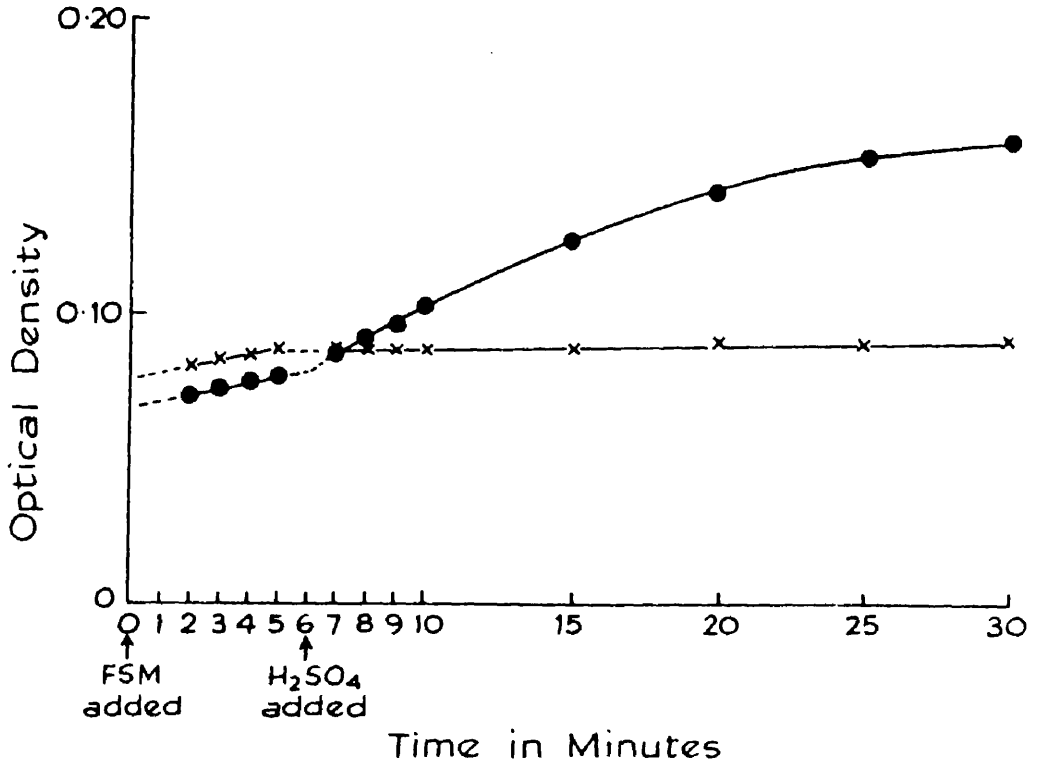


Figure 7

(a) The Effect of Different Amounts of H_2SO_4 on the Apparent Rate of Hydrolysis of Phosphocreatine.

●——● 0.63 ml 5 N H_2SO_4 added (1)

x——x 1.25 ml 5 N H_2SO_4 added (2)

The figures in brackets indicate the amount of H_2SO_4 as a proportion of the amount used by Furchgott and De Gubareff (1956). Note that with twice the amount there is no apparent hydrolysis as judged by the development of colour. A mixture of inorganic phosphate and phosphocreatine was used.

Table 2

THE INFLUENCE OF VARIOUS CONCENTRATIONS OF COPPER SULPHATE ON THE RATE OF COLOUR DEVELOPMENT IN THE ASSAY OF INORGANIC PHOSPHATE

The optical density at the different times after addition of the FSM to the inorganic phosphate solution are expressed as a percentage of the final constant optical density observed.

Time in Minutes After FSM Added	Final Concentration of $CuSO_4$ in Colorimeter Cell				
	0.00016 M	0.00032 M	0.00048 M	0.00064 M	0.0008 M
1	56	71	76	84	91
2	89	96	98	99	100
3	99	99	100	100	100
4	100	100	100	100	100
5	100	100	100	100	100

De Gubareff (1956), made a macromodification of their method feasible. This was desirable since the measurement of larger volumes of reagents incurs smaller percentage errors. With two exceptions, this was done by proportionally increasing the volumes of the reagents used. Firstly, in order that the colour development due to the presence of inorganic phosphate was complete within 2 minutes of starting the reaction, it was necessary to use a five-fold greater concentration of copper ions (Table 2). Secondly, to obtain the same apparent rate of hydrolysis of phosphocreatine as Furchgott and De Gubareff (1956), it was necessary to lower the amount of sulphuric acid added (Figure 7).

Experimental Procedure for the Estimation of Inorganic Phosphate and Phosphocreatine.

Ten ml of standard inorganic phosphate solution, standard phosphocreatine solution, a mixture of both, or brain extract were transferred to a test tube embedded in crushed ice and containing 0.4 ml of 0.02 M copper sulphate solution. A reagent blank was prepared by adding 10 ml of 0.05 M PCA to a similar

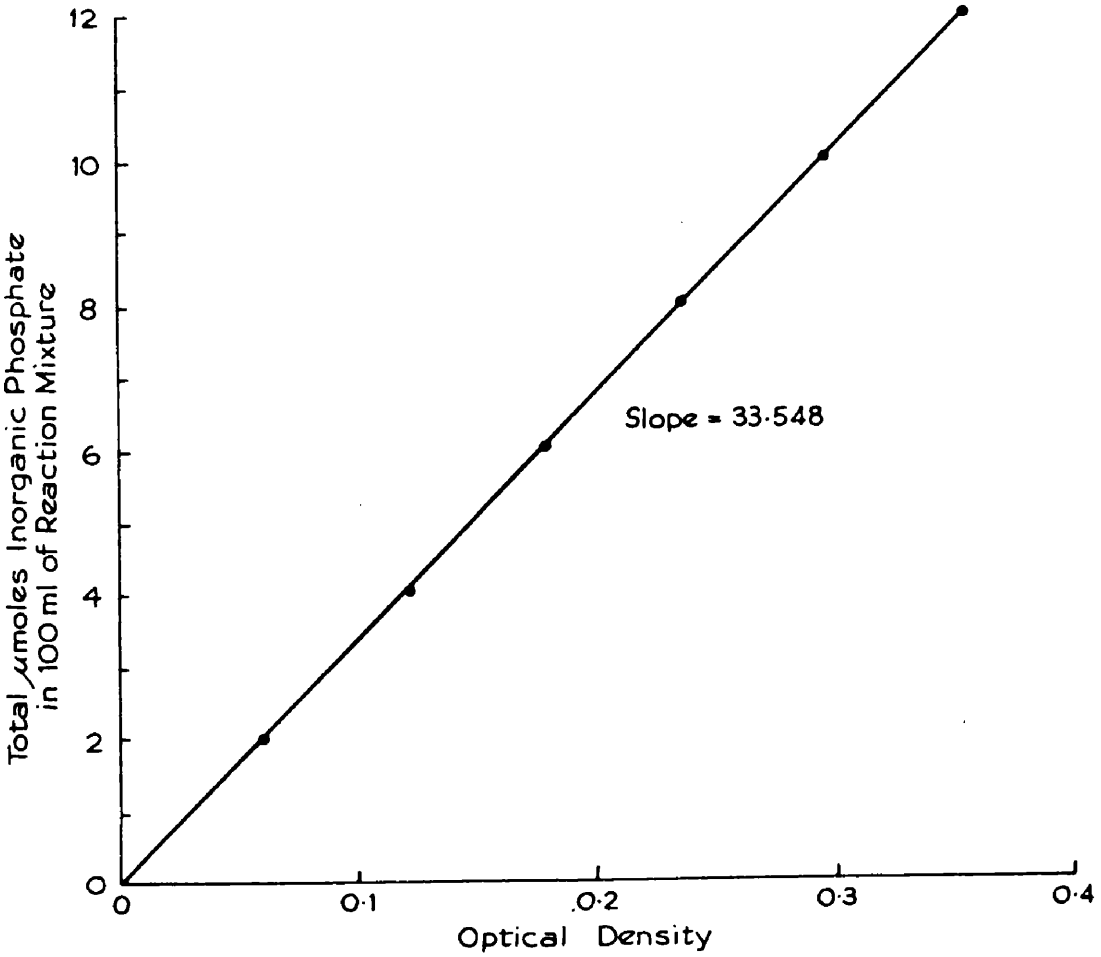


Figure 8

Calibration Curve for Inorganic Phosphate.

The optical densities were determined using a Hilger Spekker Absorptiometer fitted with Ilford number 608 filters to select a wavelength of approximately 675 mµ. Each point on the graph represents the mean of 3 determinations and a linear regression line was calculated.

Since the line passes through the origin, its slope may be used for calculating unknown concentrations of inorganic phosphate. The concentrations represent the total inorganic phosphate in 100 ml of 0.05 M perchloric acid. Ten ml aliquots were assayed as described in the text. Since the brain extract was always made up to a volume of 100 ml and 10 ml assayed similarly, the total inorganic phosphate in each brain can be directly determined from the slope of the line (e.g. 33.548 x zero time optical density of brain extract = µmoles inorganic phosphate in the whole brain).

test tube. To each of two, 8 ml capacity, 1 cm light path cells were added respectively 5 ml of the reagent blank and 5 ml of the test solution. One ml of ice-cold Fiske and Subbarow mixture (FSM) was added rapidly to each cell using a 1 ml "blow out" pipette and the contents stirred briefly with a small glass rod. The optical density of the test solution, with reference to the reagent blank, was determined 1,2,3,4, and 5 minutes after the addition of the FSM to the test solution. A Hilger Spekker Absorptiometer, fitted with Ilford number 608 filters to select a wavelength of approximately 675 mμ, was used for this purpose. At 6 minutes, 0.36 ml of 5 N sulphuric acid was delivered rapidly under the surface of the test solution using a 1 ml tuberculin syringe fitted with a fine polythene tube. The solution was again stirred and optical density readings taken each minute up to 10 minutes and thereafter at 5 minute intervals to 30 minutes.

A calibration curve (Figure 8) was constructed by estimating different concentrations of standard inorganic phosphate solutions in triplicate and

Table 3

The Estimation of Standard Solutions of Phosphocreatine.

Number of Estimations	Theoretical Phosphocreatine Concentration (µmoles/100ml of reaction mixture)	Phosphocreatine Concentration Found (µmoles/100ml of reaction mixture ± S.D.)	Per Cent Error ± S.D.
14	4.65	4.35 ± 0.06	6.4 ± 1.5

The theoretical concentration was obtained by estimating the inorganic phosphate in an aliquot which had been heated to 100°C for 10 minutes in the presence of 0.05 M perchloric acid.

Each of the 14 values used for the above table was the mean of duplicate assays.

In 7 of the 14 solutions, the phosphocreatine assay was done in the presence of 5 µmoles/100ml of reaction mixture of inorganic phosphate.

plotting a linear regression line. Since the reaction is complete in 2 minutes when only inorganic phosphate is present (Figure 5), the mean 2 to 5 minute optical density readings were used for this purpose. It was shown by Furchgott & De Gubareff (1956) that the colour development during the second part of the reaction, after the addition of the sulphuric acid, was lower than during the first part of the procedure. It was, therefore, necessary to multiply the optical density due to hydrolyzed phosphocreatine by the factor of 1.06 in order that low values for phosphocreatine were not obtained. The applicability of this correction factor to the present work was confirmed by assaying aliquots of the same standard phosphocreatine solutions before and after their acid hydrolysis (100°C, 10 minutes; Figure 6; Table 3). In brain extracts, the concentration of inorganic phosphate was determined by applying the optical denisty at zero time, obtained by extrapolation of the 2 to 5 minute readings, to the calibration curve. The difference between this optical density at zero

time and that at 30 minutes was multiplied by the correction factor of 1.06 and applied to the same calibration curve to obtain the phosphocreatine concentration.

BRAIN REMOVAL AND EXTRACTION

Rats Killed by Decapitation

Preliminary experiments were conducted using a group of untreated rats and a group of rats immobilized by the intraperitoneal administration of mephenesin (200 mg/kg). The animals were killed by decapitation, the brain quickly removed, weighed, and homogenized with 25 ml of ice-cold 0.15 M PCA in an "M.S.E. micro homogenizer" (14,000 r.p.m., 1 minute). The homogenizer blades and the vortex beaker were washed successively with 10 ml and 5 ml of ice-cold distilled water and the cell debris removed from the combined washings and PCA extract by centrifugation (0°C, 10 minutes, 2200 x g). The precipitate was then re-extracted with 9 ml of ice-cold 0.15 M PCA and centrifuged as before. The supernatants were combined, made up to a volume of 100 ml with ice-cold distilled water and assayed for adenine

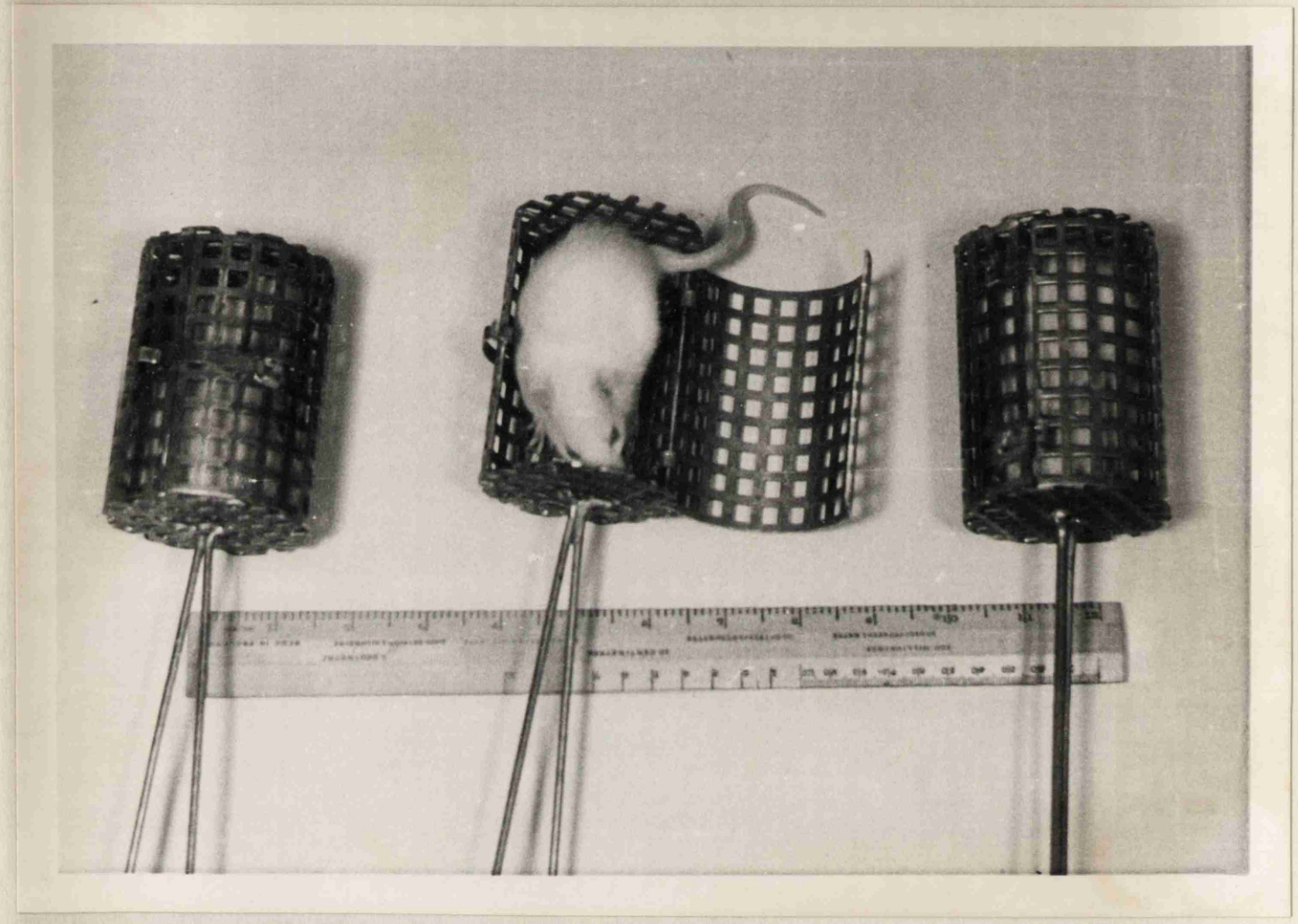


Figure 9

Photograph of the Cages in Which the Rats Were Placed After Treatment With the Drug or Control Solutions.

95g rats are shown.

Table 4

Comparison of Adenine Nucleotide Levels in the Rat Brain *in vivo* After Decapitation and Freezing Techniques.

The values for the present work are the mean of observations on 10 rats ± S.E. of the mean.

Method of Killing	μmoles per g wet weight of brain				Ratio
	AMP	ADP	ATP	AMP + ADP + ATP	$\frac{ATP}{ADP}$
1	2.09 ± 0.04	1.11 ± 0.08	0.96 ± 0.07	4.16 ± 0.08	0.92 ± 0.11
2	1.92 ± 0.05	1.19 ± 0.05	1.29 ± 0.07	4.37 ± 0.10	1.08 ± 0.07
Values of Other Workers					
3	0.26	0.57	1.77	2.60	3.10
4	0.25	0.60	1.76	2.61	2.93
5	0.46	0.99	2.58	4.03	2.61

1 - Untreated rats decapitated; 2 - Rats immobilized by intraperitoneal injection of mephesin (200 mg/kg) 10 minutes before decapitation;

3 - Doring & Gerlach (1957), rats anaesthetized, decapitated and the head immediately ffozen;

4 - Gerlach, Doring & Fleckenstein (1957), intact rat frozen;

5 - Lin, Cohen & Cohen (1958), intact rat frozen.

nucleotides.

A comparison of the values obtained using this method, with the results of other workers (Table 4), indicated that this method gave comparatively low ATP values and high ADP and AMP values. Attention was, therefore, directed towards the method of killing the rats by immersion in liquid nitrogen. Rapid fixation by this means is believed to minimize the breakdown of brain high energy phosphates and yield more realistic values (Heald, 1960).

Rats Killed by Immersion in Liquid Nitrogen

Male Wistar strain rats weighing between 75 and 95 g were used because it is difficult to fix rapidly the brains of animals over 100 g in weight in liquid nitrogen (Heald, 1960). Immediately after treatment with the drug or control solution (see page 86) each rat was placed in a small cylindrical cage (Figure 9). At the end of the experimental time ($\frac{1}{2}$, $1\frac{1}{2}$, 3, 4, 6, or 12 hours), the cage containing the rat was quickly immersed in liquid nitrogen and kept there for 3 to 5 minutes. The frozen rat was then removed from the

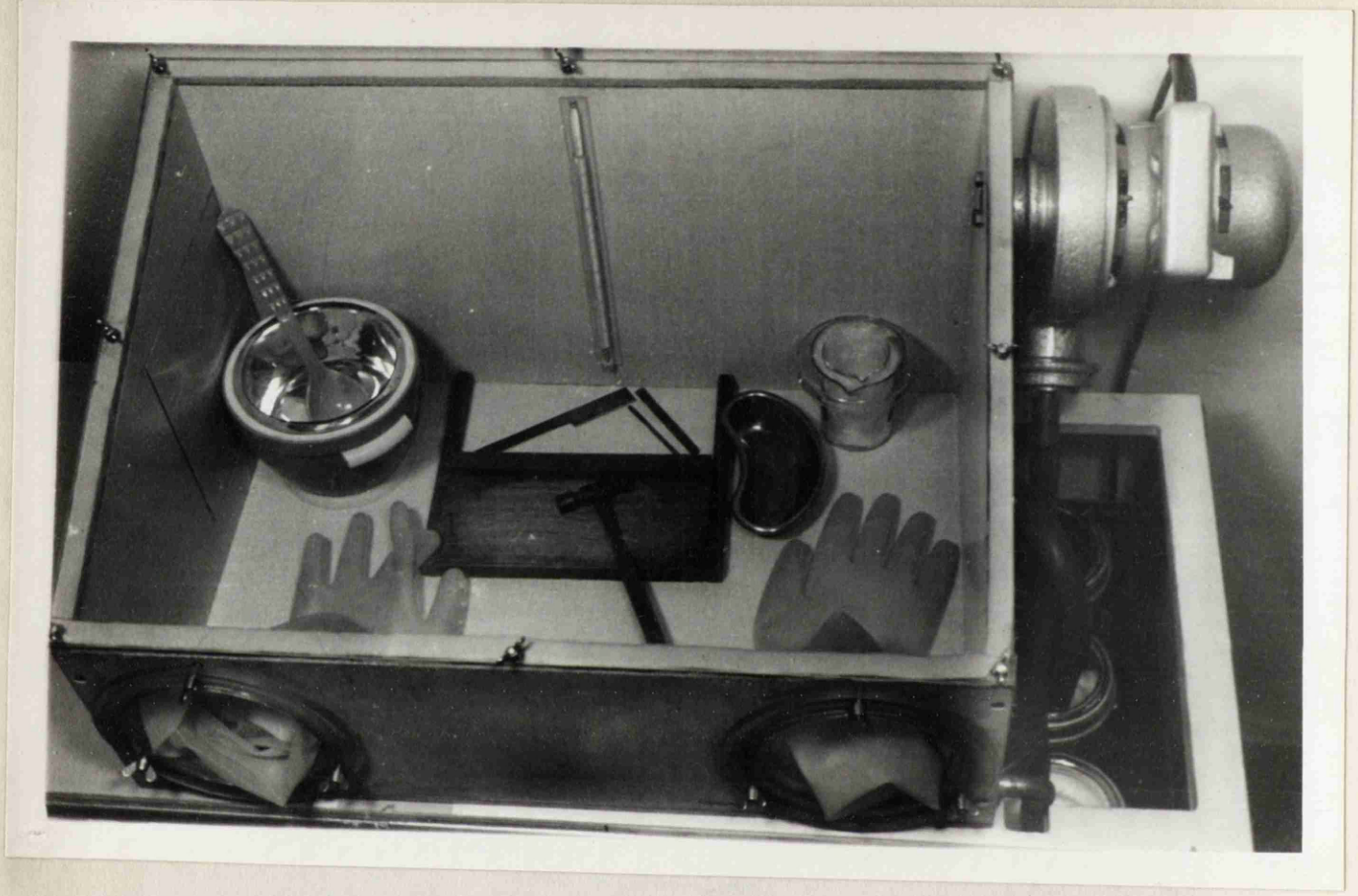


Figure 10

Photograph of the Glove Box in Which Rat Brains Were Removed From the Heads.

Air is withdrawn from the box by the blower and circulated through a water trap and two copper "U" tubes, surrounded by solid carbon dioxide and methanol, back into the box. This maintains the temperature inside the box at -5°C or lower. Note large ice-bowl which is filled with liquid nitrogen and used to keep the head frozen at all times and the small covered insulated beaker containing liquid nitrogen where the brain is placed when the dissection is completed. The chisels, hammer, and dissection board are also shown.

cage and decapitated with a large chisel. The head was then replaced in liquid nitrogen.

Removal of the brain was carried out in a glove box maintained at or below -5°C (Figure 10). A large chisel made from a high-speed power hack saw blade (cutting edge about 5 cm long) and two small chisels made from tool-steel (cutting edges about 3 and 9 mm) were found to be the most satisfactory for this procedure. Using a small tool-steel chisel, the bone was first chipped from the posterior aspect of the head to expose the posterior portion of the cerebellum and medulla oblongata. Using the large chisel, the bone was removed from the lateral aspects of the brain by making two cuts parallel to the midline approximately through the centre of each eye. Any small amounts of bone left on the lateral aspects of the brain were carefully removed with a small chisel and the top of the cranium prized off. The large chisel was then placed at right angles to the midline, just anterior to the cerebral hemispheres, and lightly tapped with a small hammer. This usually caused the

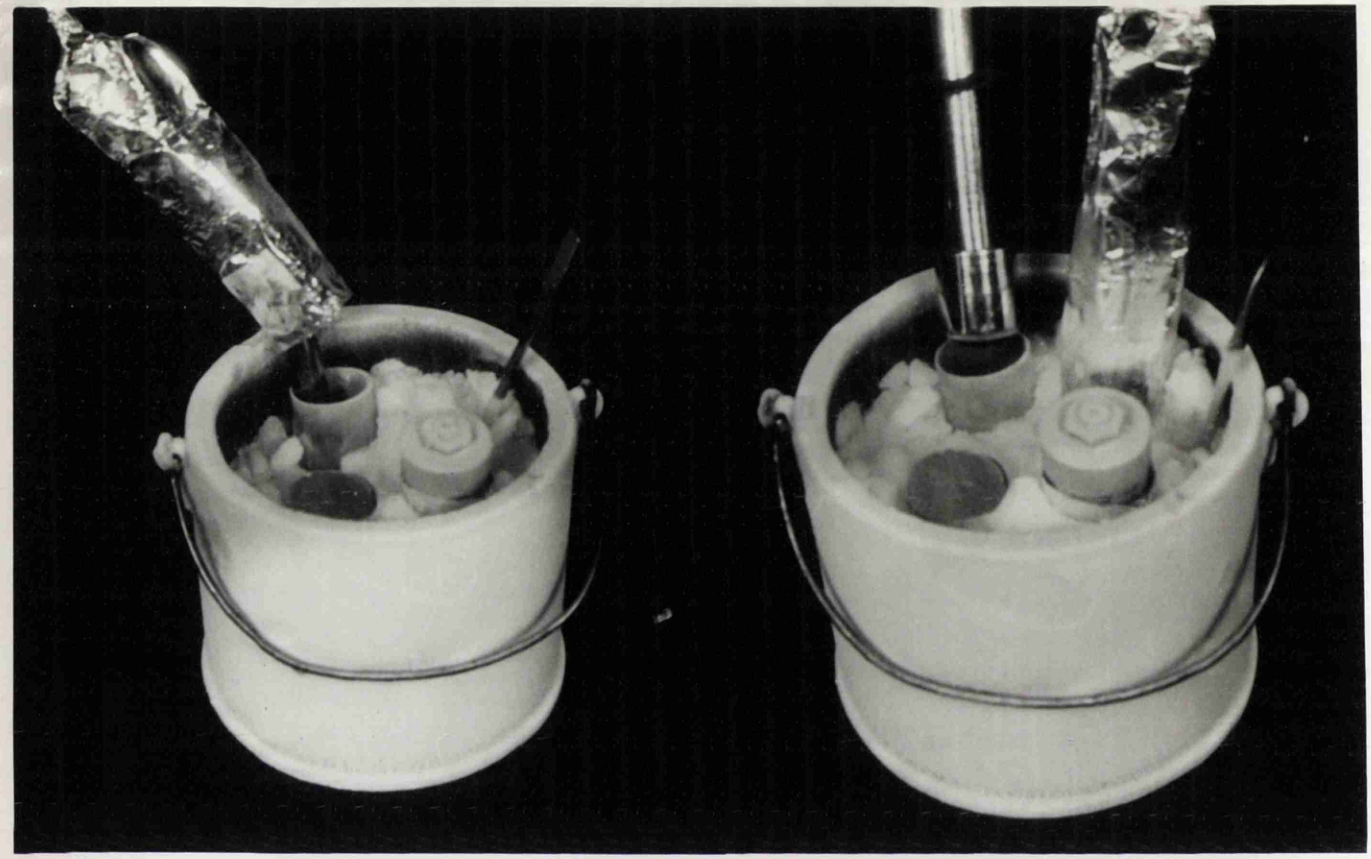


Figure 11

Photograph of Apparatus Used for Pulverizing the Frozen Brain.

A stainless steel pestle is chilled by placing it in a 50 ml stainless steel centrifuge tube embedded in solid carbon dioxide (top left). An aluminium foil cover is placed over the pestle to prevent frost forming on it; this is shown partly removed on left and completely removed on right. Two other stainless steel centrifuge tubes are placed in aluminium foil casings embedded in the solid carbon dioxide to chill and are used for pulverizing the brain; in the photograph only one centrifuge cup is in place. For the pulverization, about 25 ml of liquid nitrogen is placed in one of the centrifuge tubes. The brain is then placed in this and the pestle put gently on top of it. As soon as the liquid nitrogen has boiled off, the rubber washer around the shaft of the pestle is pressed firmly down on top of the centrifuge tube and the shaft struck several sharp blows with a hammer. The small stainless steel spatula is used to scrape any adhering pieces of brain off the pestle back into the tube and to scrape the bottom of the tube to make sure that the powdered brain is not caked to the bottom of it.

whole brain, with the exception of the olfactory lobes, to come loose from the remainder of the skull. The brain was carefully examined and any small pieces of adhering bone removed. It was then transferred to an insulated beaker containing clean liquid nitrogen. During the removal of the bone from the cerebellum, the parafloculi were lost. Throughout the dissection the brain was frequently replaced in liquid nitrogen and the chisels and forceps used to hold it also chilled in this coolant. Finally, the brain was transferred from the beaker of liquid nitrogen to a torsion balance, the pan of which was chilled in liquid nitrogen, weighed, and quickly placed in a Dewar flask of liquid nitrogen where it was stored until extracted (up to 24 hours).

Extraction of the Brain

The frozen brain was first pulverized in a stainless steel centrifuge tube as described by Furchgott and De Gubareff (1956). The apparatus shown in Figure 11 was used for this purpose. A small stainless steel spatula, chilled to the

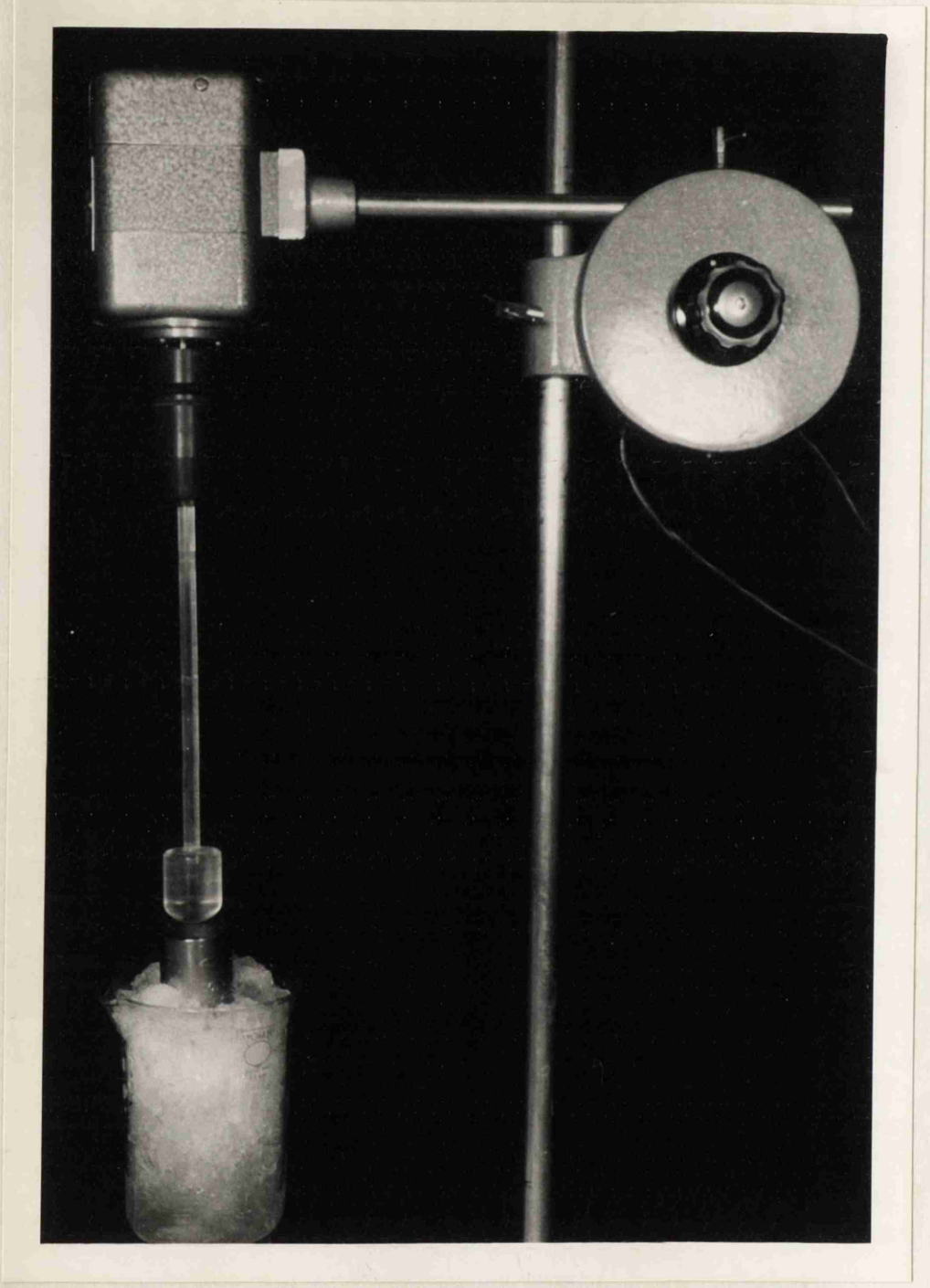


Figure 12

Photograph of the Apparatus Used for Homogenizing the Brain.

The stainless steel centrifuge tube is embedded in crushed ice. The pestle and shaft are plastic and are chilled to 0°C before use.

temperature of solid carbon dioxide, was used to scrape the bottom of the centrifuge tube to ensure that the powdered brain was not caked to the tube. The centrifuge tube was then transferred to a beaker of crushed ice and allowed to stand 2 minutes. This delay was found necessary to prevent freezing of the PCA on its addition to the chilled tube. Then 10 ml of ice-cold 0.3 M PCA was quickly added with stirring. To ensure rapid penetration of the PCA into small adhering pieces of brain, the tissue was immediately homogenized for 2 minutes using a loosely fitting plastic pestle (2000 r.p.m.; Figure 12). The suspension was centrifuged (0°C, 6 minutes, 2200 x g) and the supernatant filtered at 5°C through a plaited 9 cm diameter Whatman number 1 filter paper into a 100 ml volumetric flask packed in crushed ice. The tissue was re-extracted with 6.7 ml of ice-cold 0.3 M PCA, stirred for 2 minutes, and centrifuged as before. The second extract was filtered and combined with the first. After each filtration, the filter paper was washed with approximately 10 ml of ice-cold triple distilled water. The volumetric flask was then made

DRUGS EMPLOYED

(c) Other antidepressives and related compounds.

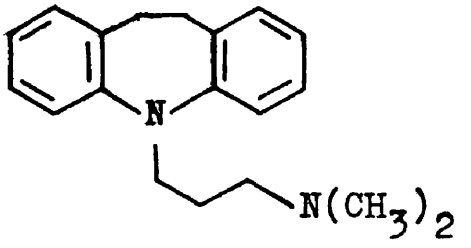
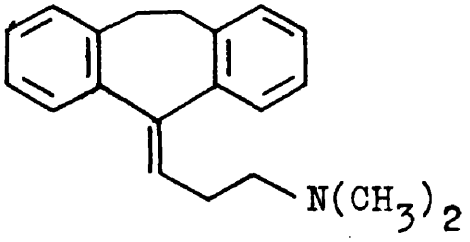
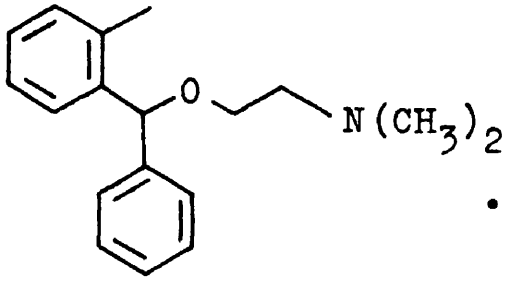
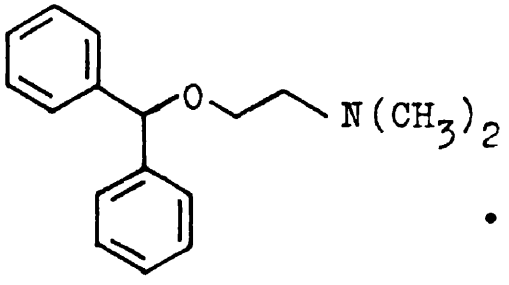
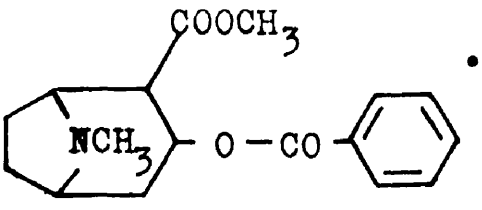
Structural formula and approved name	Enantiomorph employed	Source
 • HCl IMIPRAMINE		Geigy Pharmaceutical Co. Ltd.
 • HCl AMITRIPTYLINE		Merck Sharpe and Dohme Ltd.
 • HCl ORPHENADRINE	racemate	Camden Chemical Co. Ltd.
 • HCl DIPHENHYDRAMINE		Parke Davis and Co.
 • HCl Cocaine	<u>laevo</u>	The British Drug Houses Ltd.

Table 5

DRUGS EMPLOYED

(b) Monoamine oxidase inhibitors and related compounds.

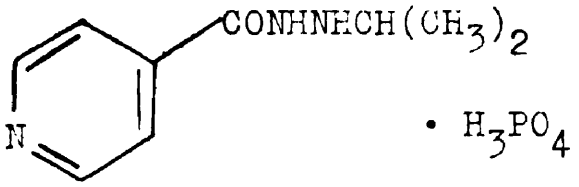
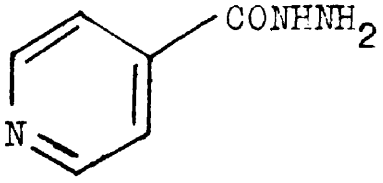
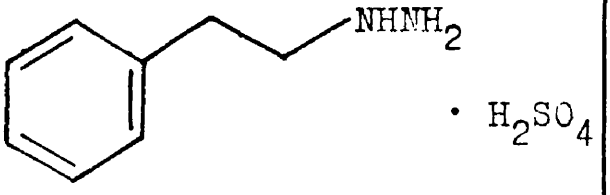
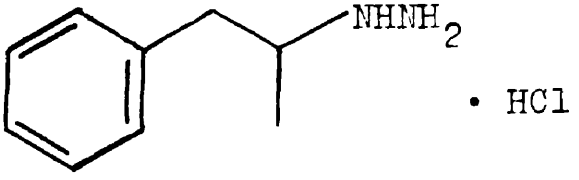
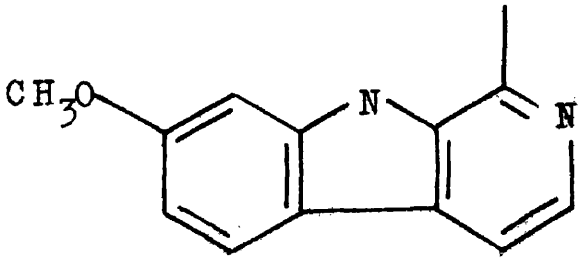
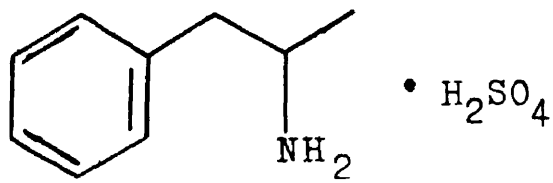
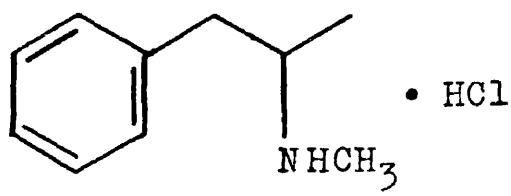
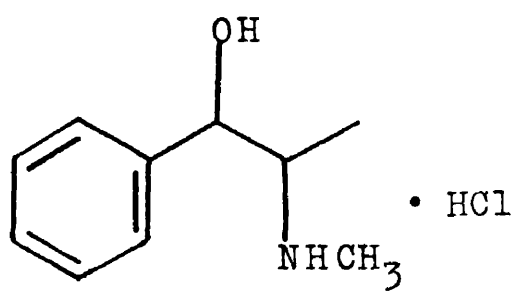
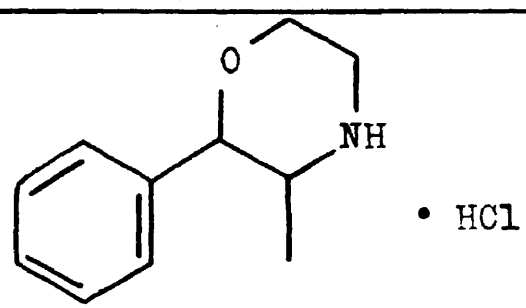
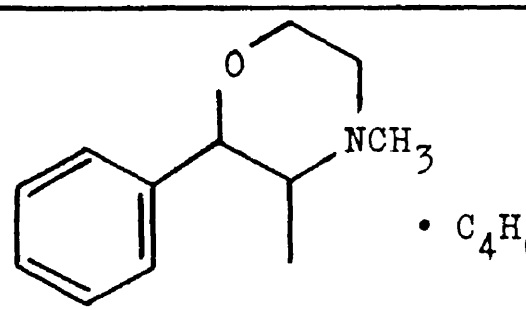
Structural formula and approved name	Enantiomorph employed	Source
 IPRONIAZID		Roche Products Ltd.
 ISONIAZID		Smith and Nephew Research Ltd.
 PHENELZINE		William R. Warner and Co. Ltd.
 PHENIPRAZINE	racemate	Benger Laboratories Ltd.
 TRANYLCYPROMINE	racemate	Smith Kline and French Laboratories Ltd.
 HARMINE · HCl		L. Light and Co. Ltd.

Table 5

DRUGS EMPLOYED

(a) Amphetamine-like central nervous system stimulants

Structural formula and approved name	Enantiomorph employed	Source
 • H_2SO_4 AMPHETAMINE	dextro laevo	Smith, Kline & French Laboratories Ltd.
 • HCl METHYLAMPHETAMINE	dextro	Burroughs Wellcome & Co.
 • HCl EPHEDRINE	laevo	British Drug Houses Ltd.
 • HCl PHENMETRAZINE	racemate	Pfizer Ltd.
 • $C_4H_6O_4$ PHENDIMETRAZINE	dextro laevo	Ayerst, Mckenna & Harrison Ltd.

up to the mark with this solvent to produce a final concentration of PCA of approximately 0.05 M. Two 10 ml aliquots of this extract were immediately taken for the assay of adenine nucleotides, and inorganic phosphate and phosphocreatine, respectively.

PREPARATION OF SOLUTIONS OF DRUGS USED

The structural formulae, approved names, salt used, optical rotation (if any), and sources of the drugs used in this investigation are shown in Table 5. Each drug was dissolved in 0.9 per cent sodium chloride and the solution sterilized. With the exception of the solutions of dextro- and laevo-amphetamine and methylamphetamine, which were sterilized in an autoclave, the drug solutions were sterilized by filtration and kept in sterile multi-dose containers. Although an attempt was made to make the concentration of all drug solutions such that the administration of 0.2 ml per 100 g body weight would give the desired dose, the low solubility of harmine prevented this. Thus, the 20 mg/kg doses of harmine and cocaine were given in 0.4 ml/100 g body weight and the 10 and 40 mg/kg doses

of harmine were given in 1.0 ml/100g body weight. In all cases the drug and control (an equivalent volume of sterile 0.9% solium chloride) solutions were administered by intraperitoneal injection using all glass tuberculin syringes and stainless steel needles previously sterilized by heat.

STATISTICAL DESIGN AND ANALYSIS

Three main considerations were involved in devising a statistical design for these experiments. First, since the experiments were carried out *in vivo*, there was the possibility of the results being affected by variation between different animals. Second, it was possible that various degrees of breakdown of the highly labile phosphate esters might occur during the slightly different times of storage of different brains in the liquid nitrogen. Third, differences in technique during a series of experiments could, it was believed, possibly affect the results obtained. In an attempt, therefore, to minimize the likelihood of these sources of variation biasing the results, the order of treatment with the drug and control

solutions and all subsequent procedures were randomized.

The following methods were used for the randomization. Groups of 2 (series 1 and 9) or 3 (all other series) rats of equal body weight were selected from stock. One rat in each group served as a control. Two methods of randomizing the order of treatment of the rats in each group were used. In series 1 and 2 this was accomplished by using a table of random numbers and assigning equal chances for each treatment to come first or second (series 1) or first, second or third (series 2) in each group. In all other series, except 9 and 10, three 3 x 3 latin squares were used to effect the randomization. In series 9 and 10 a double randomization was used. For each time interval in series 9, the same type of statistical design was used as in series 1. The statistical design for each time interval in series 10 was three, 3 x 3 latin squares. In order that a direct comparison could be made between the three time intervals in these two series, the order in which the groups from the different times were done was randomized

using three, 3 x 3 latin squares. In all cases, the order of killing the rats, removal of the brain, extraction, and assay of the brain phosphates followed the order of treatment with the drug or control solutions.

The data thus obtained was evaluated employing the analysis of variance appropriate to the statistical design used (Snedecor, 1956). Samples of the different types of statistical designs used and their analysis, and one applied experimental protocol, are shown in appendix II.

RESULTS

RESULTS

Amphetamine-like Central Nervous System Stimulants

Gross Observations

Although no attempt was made to measure the degree to which the drugs used in the present experiments stimulated the central nervous system, it was obvious from the behaviour of the rats that some produced greater stimulation than others. In all the experiments the control rats soon settled in the small cages and were comparatively inactive. Within 15 to 30 minutes of being placed in the cage they were usually dozing or sleeping. Thereafter they would from time to time wake up, turn around in the cage, and then settle and go back to sleep. It was thus a matter of chance whether they were awake or asleep when they were plunged into the liquid nitrogen. In contrast, the rats which had received d-amphetamine (0.45, 0.90, or 2.5 mg/kg) or d-methylamphetamine (0.45 or 0.90 mg/kg) generally remained awake and alert up to the time when they were killed ($\frac{1}{2}$, $1\frac{1}{2}$, 3 and 4 hours after treatment). They turned around in their

Table 6

In Vivo Effect of Amphetamine and Related Compounds on the Adenine Nucleotide, Inorganic Phosphate and Phosphocreatine Concentrations in the Rat Brain.

All values are the means ± S.E. of mean obtained 4 hours after injection of the drug.

Series	Treatment	Dose (mg/kg)	No. of Rats	Concentration in μmoles per g Frozen Tissue						Ratio
				Inorganic Phosphate	Phospho-creatine	AMP	ADP	ATP	AMP + ADP + ATP	$\frac{ATP}{ADP}$
1	Control		10	-	-	0.82 ± 0.04	1.14 ± 0.12	1.91 ± 0.09	3.88 ± 0.08	1.79 ± 0.20
	d-Methylamphetamine	0.45	10	-	-	0.88 ± 0.07	†0.75 ± 0.07	‡2.41 ± 0.07	4.03 ± 0.09	‡3.31 ± 0.31
	Control		10	-	2.99 ± 0.12	0.85 ± 0.07	0.94 ± 0.05	2.11 ± 0.06	3.91 ± 0.11	2.32 ± 0.17
2	l-Amphetamine	0.45	10	-	3.21 ± 0.10	0.81 ± 0.04	0.98 ± 0.07	2.10 ± 0.06	3.89 ± 0.07	2.29 ± 0.25
	d-Amphetamine	0.45	10	-	3.05 ± 0.17	0.86 ± 0.05	*0.78 ± 0.05	†2.39 ± 0.05	4.03 ± 0.10	*3.22 ± 0.23
	Control		9	7.48 ± 0.93	3.17 ± 0.18	0.85 ± 0.05	1.04 ± 0.04	1.96 ± 0.05	3.84 ± 0.09	1.91 ± 0.10
	d-Methylamphetamine	0.90	9	7.08 ± 0.83	3.82 ± 0.14	0.87 ± 0.03	0.85 ± 0.05	*2.30 ± 0.11	4.02 ± 0.09	*2.82 ± 0.31
	d-Amphetamine	0.90	9	6.91 ± 0.88	3.08 ± 0.12	0.83 ± 0.03	0.90 ± 0.05	*2.25 ± 0.06	3.99 ± 0.07	*2.56 ± 0.16
	Control	0.90	9	-	3.41 ± 0.17	0.81 ± 0.09	1.01 ± 0.07	2.04 ± 0.08	3.86 ± 0.17	2.12 ± 0.21
4	l-Ephedrine	0.90	9	-	2.94 ± 0.14	0.79 ± 0.07	‡0.83 ± 0.07	1.99 ± 0.09	3.62 ± 0.15	2.54 ± 0.30
	dl-Phenmetrazine	0.90	9	-	3.55 ± 0.20	0.90 ± 0.07	‡0.63 ± 0.04	2.24 ± 0.05	3.77 ± 0.11	†3.70 ± 0.26
	Control		9	8.61 ± 1.11	3.42 ± 0.13	0.77 ± 0.04	0.86 ± 0.07	1.99 ± 0.05	3.62 ± 0.07	2.41 ± 0.19
5	l-Ephedrine	1.80	9	8.62 ± 0.89	3.30 ± 0.09	0.63 ± 0.06	0.91 ± 0.04	1.86 ± 0.09	3.40 ± 0.10	2.09 ± 0.17
	dl-Phenmetrazine	1.80	9	*5.82 ± 0.62	3.34 ± 0.14	0.74 ± 0.04	‡0.54 ± 0.03	†2.24 ± 0.07	3.52 ± 0.09	‡4.28 ± 0.32
	Control		9	4.68 ± 0.71	3.84 ± 0.11	0.73 ± 0.05	0.68 ± 0.04	2.22 ± 0.06	3.63 ± 0.08	3.34 ± 0.25
6	l-Amphetamine	10.0	9	4.17 ± 0.60	3.72 ± 0.13	†0.53 ± 0.03	‡0.39 ± 0.03	‡2.78 ± 0.05	3.70 ± 0.06	‡7.52 ± 0.69
	l-Ephedrine	20.0	9	4.27 ± 0.34	3.71 ± 0.10	†0.55 ± 0.03	‡0.51 ± 0.03	‡2.69 ± 0.05	3.75 ± 0.08	‡5.35 ± 0.31
	Control		9	5.23 ± 0.46	3.62 ± 0.15	0.77 ± 0.04	0.80 ± 0.03	2.32 ± 0.05	3.89 ± 0.09	2.93 ± 0.10
7	dl-Phenmetrazine	1.80	9	5.20 ± 0.53	3.65 ± 0.12	*0.61 ± 0.04	‡0.44 ± 0.04	‡2.82 ± 0.06	3.87 ± 0.09	‡6.82 ± 0.65
	d-Phendimetrazine	1.80	9	4.77 ± 0.40	3.49 ± 0.12	0.71 ± 0.04	0.71 ± 0.06	2.48 ± 0.06	3.88 ± 0.11	3.80 ± 0.33
	Control		9	4.01 ± 0.21	3.57 ± 0.17	0.72 ± 0.03	0.76 ± 0.03	2.13 ± 0.05	3.62 ± 0.07	2.82 ± 0.12
8	d-Phendimetrazine	3.60	9	4.95 ± 0.51	3.48 ± 0.13	0.64 ± 0.03	†0.49 ± 0.03	‡2.63 ± 0.07	3.76 ± 0.07	‡5.59 ± 0.37
	l-Phendimetrazine	3.60	9	4.93 ± 0.63	3.60 ± 0.15	0.78 ± 0.04	0.71 ± 0.02	2.25 ± 0.06	3.74 ± 0.07	3.18 ± 0.12

§ One missing value calculated. Significance of differences from control: *0.05 > P > 0.01 †0.01 > P > 0.001 ‡0.001 > P.

cages quite frequently and repeatedly washed themselves. This increase in motor activity seemed to be most marked after 2.5 mg/kg of d-amphetamine. The higher doses of dl-phenmetrazine (1.80 mg/kg), d-phendimetrazine (3.60 mg/kg), l-amphetamine (10 mg/kg) and l-ephedrine (20 mg/kg) produced effects similar to those of the lower doses of d-amphetamine and d-methylamphetamine. The lower doses of l-amphetamine (0.45 mg/kg), l-ephedrine (0.90 mg/kg), d-phendimetrazine (1.80 mg/kg) and l-phendimetrazine (3.60 mg/kg) produced little effect on the behaviour of the rats.

Effects on Brain Levels of Adenine Nucleotides, Inorganic Phosphate and Phosphocreatine.

The levels of adenine nucleotides, inorganic phosphate and phosphocreatine in the brain 4 hours after injection of the control solution, d-methylamphetamine, d- and l-amphetamine, l-ephedrine, dl-phenmetrazine and d- and l-phendimetrazine are shown in Table 6. In the doses employed, none of these drugs caused any significant change in the level of phosphocreatine or in the total adenine nucleotide content (AMP + ADP + ATP) of the brain. Although the inorganic phosphate level

appeared to be lower after treatment with most of these drugs, only the decrease observed after 1.80 mg/kg of dl-phenmetrazine was statistically significant (series 5). Since this effect was not observed with the same dose of dl-phenmetrazine in series 7, no importance is attached to this isolated observation. Similarly, there was only a significant fall in the AMP level after l-amphetamine (10 mg/kg), l-ephedrine (20 mg/kg; series 6) and dl-phenmetrazine (1.80 mg/kg; series 7).

With the exception of l-phendimetrazine, after a sufficiently high dose of all of these drugs there was a significant increase in the ATP level and a significant decrease in the ADP level. These changes were reflected by a marked and significant increase in the ATP/ADP ratio. The dose of each of these drugs required to produce this effect seemed to correspond to the dose which produced gross signs of central nervous system stimulation in the present experiments and which has been reported by other workers to cause hypermotility (Brown & Searle, 1938; Schulte et al., 1941; Garberg & Sandberg, 1960). Using the ratio of ATP/ADP as an

index, it is immediately obvious from series 2 that the laevo isomer of amphetamine is less potent than the dextro isomer. Comparing series 1 and 2, d-methylamphetamine appears to be slightly more potent than d-amphetamine. From series 1 and 4 it can be seen that 0.90 mg/kg dl-phenmetrazine produced about the same increase in the ATP/ADP ratio as 0.45 mg/kg d-methylamphetamine. It is apparent from series 4 and 5 that l-ephedrine is less potent than d-methylamphetamine, d-amphetamine and dl-phenmetrazine. However, after high doses of l-amphetamine (10 mg/kg) and l-ephedrine (20 mg/kg), which produced signs of stimulation, the ATP/ADP ratio was increased. From series 7 and 8, it is apparent that d-phendimetrazine is less potent than dl-phenmetrazine and the laevo isomer of phendimetrazine is less potent than the dextro isomer. Thus on the basis of an ability to increase the ATP/ADP ratio, d-methylamphetamine is the most potent compound of this group. The dextro isomer of amphetamine would appear to be slightly less potent than dl-phenmetrazine. But 0.45 mg/kg of d-amphetamine produced a significant increase in the ATP level and 0.90 mg/kg dl-phenmetrazine

Table 7

Series 9: In Vivo Effect of Methylamphetamine (0.90 mg/kg) on the Adenine Nucleotide, Inorganic Phosphate and Phosphocreatine Concentration in the Rat Brain at Various Times After Treatment.

All values are the mean of observations on 9 rats ± S.E. of mean.

Treatment	Time after Treatment (hr.)	Concentration in μmoles per g Frozen Tissue						Ratio
		Inorganic Phosphate	Phospho-creatine	AMP	ADP	ATP	AMP + ADP + ATP	ATP / ADP
Control	½	6.24 ± 0.69	3.70 ± 0.17	0.89 ± 0.06	0.92 ± 0.05	2.09 ± 0.05	3.90 ± 0.06	2.33 ± 0.17
Methylamphetamine	½	5.58 ± 0.96	3.77 ± 0.17	0.92 ± 0.07	†0.78 ± 0.04	‡2.19 ± 0.05	3.89 ± 0.08	†2.89 ± 0.19
Control	1½	6.22 ± 0.61	3.66 ± 0.25	0.83 ± 0.04	0.82 ± 0.02	2.10 ± 0.05	3.74 ± 0.06	2.58 ± 0.12
Methylamphetamine	1½	7.16 ± 0.93	†3.24 ± 0.16	0.90 ± 0.06	0.75 ± 0.03	‡2.30 ± 0.05	†3.96 ± 0.07	‡3.08 ± 0.08
Control	3	7.51 ± 1.12	3.73 ± 0.19	0.87 ± 0.08	0.90 ± 0.03	2.05 ± 0.04	3.82 ± 0.09	2.32 ± 0.13
Methylamphetamine	3	8.75 ± 1.14	3.58 ± 0.21	0.84 ± 0.06	‡0.71 ± 0.04	‡2.39 ± 0.07	3.94 ± 0.09	‡3.44 ± 0.22

Significance of differences from control at the same time interval:

†0.01 > P > 0.001 ‡0.001 > P

did not. Considering the effects on the levels of ADP and ATP as well as the ratio of ATP/ADP, the data indicate that d-amphetamine is slightly more potent that dl-phenmetrazine. The next most potent compound in the group is probably d-phendimetrazine, although 3.60 mg/kg l-amphetamine was not tested. Since the magnitude of the change in the ADP and ATP levels was not as great with 20 mg/kg l-ephedrine as with 10 mg/kg l-amphetamine, l-ephedrine is probably the least potent member of the group, although again, doses of l-phendimetrazine higher than 3.60 mg/kg were not tested.

The levels of adenine nucleotides, inorganic phosphate and phosphocreatine $\frac{1}{2}$, $1\frac{1}{2}$ and 3 hours after treatment with 0.90 mg/kg of d-methylamphetamine are shown in Table 7 (series 9; see appendix II, page xv for statistical design). There was no difference between the levels of inorganic phosphate or AMP in the drug-treated and control animals at any of the three time intervals. The phosphocreatine level was lower in the drug-treated animals after $1\frac{1}{2}$ hours. At each of the three time intervals the animals treated with d-methylamphetamine had a significantly higher level of

Table 8

Series 10: In Vivo Effect of d-Amphetamine (2.5mg/kg) and dl-Phenmetrazine (1.80mg/kg) on the Adenine Nucleotide, Inorganic Phosphate and Phosphocreatine Concentration in the Rat Brain at Various Times After Treatment.

All values are the mean of observations on 9 rats ± S.E. of mean.

Treatment	Time after Treatment (hr.)	Concentration in µmoles per g Frozen Tissue						Ratio
		Inorganic Phosphate	Phospho-creatine	AMP	ADP	ATP	AMP + ADP + ATP	ATP/ADP
Control	½	6.11 ± 0.91	3.47 ± 0.19	0.77 ± 0.03	0.73 ± 0.02	2.26 ± 0.08	3.76 ± 0.11	3.13 ± 0.15
d-Amphetamine	½	4.66 ± 0.37	3.96 ± 0.38	‡0.60 ± 0.05	‡0.48 ± 0.05	‡2.86 ± 0.06	3.94 ± 0.07	†6.64 ± 0.85
dl-Phenmetrazine	½	4.21 ± 0.18	3.81 ± 0.19	‡0.58 ± 0.05	‡0.46 ± 0.02	‡2.68 ± 0.05	3.72 ± 0.12	†5.84 ± 0.16
Control	1½	4.70 ± 0.62	3.99 ± 0.09	0.80 ± 0.03	0.74 ± 0.03	2.30 ± 0.07	3.84 ± 0.10	3.15 ± 0.17
d-Amphetamine	1½	4.23 ± 0.12	3.86 ± 0.15	*0.63 ± 0.08	†0.46 ± 0.04	‡2.86 ± 0.05	3.95 ± 0.10	†6.71 ± 0.75
dl-Phenmetrazine	1½	5.24 ± 0.68	3.81 ± 0.15	*0.56 ± 0.03	†0.47 ± 0.04	‡2.84 ± 0.07	3.87 ± 0.05	†6.34 ± 0.53
Control	3	5.10 ± 0.41	4.23 ± 0.19	0.74 ± 0.02	0.71 ± 0.04	2.30 ± 0.09	3.75 ± 0.10	3.31 ± 0.22
d-Amphetamine	3	4.62 ± 0.56	3.88 ± 0.13	‡0.58 ± 0.03	‡0.36 ± 0.03	‡2.95 ± 0.07	3.89 ± 0.07	‡8.54 ± 0.66
dl-Phenmetrazine	3	4.38 ± 0.42	4.04 ± 0.21	‡0.53 ± 0.03	‡0.42 ± 0.05	‡2.79 ± 0.07	3.74 ± 0.05	‡7.73 ± 1.20

Significance of differences from control at the same time interval:

*0.05 > P > 0.01 †0.01 > P > 0.001 ‡0.001 > P

ATP and a significantly higher ATP/ADP ratio. The drug-treated animals also had a lower level of ADP but this difference was only significant at the ½ hour and 1½ hour time intervals. In both the control and drug-treated animals the levels of ATP and ADP, and the ATP/ADP ratio did not alter significantly in the course of the experiment (i.e. no significance between ½, 1½ and 3 hour values).

Using a similar statistical design (see appendix II, page xvii), the effects at different times after treatment with d-amphetamine (2.5 mg/kg) and dl-phenmetrazine were also studied (Table 8; series 10). There was no difference between the levels of inorganic phosphate, phosphocreatine or in the total adenine nucleotide content (AMP + ADP + ATP) in the drug-treated and control animals at any of the three time intervals. At all three time intervals, however, the animals treated with both drugs had a significantly higher level of ATP and a significantly higher ATP/ADP ratio, and significantly lower levels of ADP and AMP. Again, during the course of the experiment the values observed for each group of animals (i.e. control,

d-amphetamine and dl-phenmetrazine) showed no significant change with time. It should be noted that a fall in the AMP level was also observed 4 hours after treatment with 1.80 mg/kg dl -phenmetrazine (Table 6; series 6 & 7), and 10 and 20 mg/kg respectively of l-amphetamine and l-ephedrine (Table 6; series 6 & 7. These same doses of l- and d-amphetamine and l-ephedrine, which in the present experiments caused a fall in the AMP level as well as a fall in the ADP level and rises in the ATP level and the ATP/ADP ratio, were those reported to be the most effective so far as their ability to increase the motility of rats was concerned (Schulte et al., 1941)

The experiments of series 9 (Table 7) and 10 (Table 8) together with the data in Table 6 show, therefore, that the same qualitative effects on ATP and ADP levels in the brain occur ½, 1½, 3 and 4 hours after treatment with d-methylamphetamine, d-amphetamine and dl-phenmetrazine. At all of these times the rats treated with these drugs show behavioural signs of central nervous system stimulation.

Table 9

The Comparative Ability of Iproniazid and Isoniazid to Diminish the Normal Sleeping Time in the Rat.

Each value is the mean of 6 observations ($\pm$ S.E. of mean). The rats were selected in groups of 3 of equal body weight (80 - 90 g) and the order of treatment was randomized using two 3 x 3 latin square designs. Each rat was placed in a small cylindrical cage. The time in minutes that each rat stayed awake during the period 1 to 3 hours after treatment was recorded by an observer who did not know what treatment each rat had received.

Control	Iproniazid (40 mg/kg)	Isoniazid (40 mg/kg)
50.5 $\pm$ 9.9	* 93.0 $\pm$ 8.9	* 90.8 $\pm$ 9.2

Significance of differences from control:

$* \; 0.05 > P > 0.01$

There was no significant difference between Iproniazid and Isoniazid.

Monoamine Oxidase Inhibitors and Related Compounds

Gross Observations.

In general, the drugs in this group produced less marked effects on the behaviour of the rats than did amphetamine. Compared to the control rats, those treated with iproniazid and phenelzine appeared to be more alert and seemed to sleep less. Since isoniazid is not used as an antidepressive drug and is not normally considered to be one, it was at first surprising to note that the rats treated with it appeared little, if any, different from those treated with iproniazid. That is, rats treated with both iproniazid and isoniazid stayed awake significantly longer during the period 1 to 3 hours after treatment than did the controls (Table 9). Isoniazid has been reported to produce central nervous system stimulation in patients being treated for tuberculosis (Salzer & Luve, 1953). The animals treated with pheniprazine and tranylcypromine were not only more alert than the controls, especially with higher doses, but they also seemed to turn around in their cages more often.

These observations agree with reports that these two drugs produce effects similar to amphetamine but less intense in nature (Horita, 1959; Tedeschi et al., 1959; Spector et al., 1960). The gross effects produced by both pheniprazine and tranylcypromine began about ½ hour after injecting them and were quite marked 3 and 6 hours after treatment. Twelve hours after treatment, the pheniprazine-treated rats appeared similar to the controls while the tranylcypromine-treated rats seemed to be still slightly more alert. In contrast, the short acting monoamine oxidase inhibitor, harmine, produced quite different effects. Within about 5 minutes of an intraperitoneal injection, a tremor developed. Although with lower doses the tremor seemed to be intermittent, the application of external stimuli such as snapping the fingers or simply touching the rat caused the tremor to become quite violent again. These effects were most marked when 40 mg/kg of harmine was given, and in this case, the tremor continued almost continuously for about ¾ of an hour. The effect seemed to disappear 1 to 2 hours after treatment. Harmine also has been reported to produce a tremor in

the mouse (Zetler, 1957). Cocaine produced signs of stimulation similar to amphetamine, but less marked, and the effect seemed largely to disappear about 2 hours after treatment.

Effects on Brain Levels of Adenine Nucleotides, Inorganic Phosphate and Phosphocreatine.

The doses of the long acting monoamine oxidase inhibitors used in the present study (phenelzine, 5 mg/kg; iproniazid, 14, 20 & 40 mg/kg; pheniprazine, 1 & 2 mg/kg; tranylcypromine, 2 & 8 mg/kg) have been reported to produce 50 to 100 per cent inhibition of monoamine oxidase in the rat brain in vivo (Horita, 1961; Green & Erickson, 1960). A dose of 30 mg/kg of harmine has been reported to produce a 63 and 72 per cent increase respectively in the 5-hydroxytryptamine and noradrenaline level of the rat brain (Pletscher, Besendorf, Bachtold & Gey, 1959). Doses above and below this (10, 20 & 40 mg/kg) were used in the present study. For comparative purposes, isoniazid was used in the same doses as the chemically closely related compound, iproniazid. The doses of cocaine used were determined from preliminary experiments in which behavioural signs of stimulation

Table 10

In vivo effect of Monoamine Oxidase Inhibitors and Related Compounds on the Adenine Nucleotide, Inorganic Phosphate and Phosphocreatine Concentrations in the Rat Brain.

All values are the means of observations on 9 rats ± S.E. of mean obtained 3 hours after injection of the drug or control solutions.

Series	Treatment	Dose (mg/kg)	Concentration in µmoles per g Frozen Tissue						Ratio
			Inorganic Phosphate	Phospho-creatine	AMP	ADP	ATP	AMP + ADP + ATP	$\frac{ATP}{ADP}$
	Control	0	4.20 ± 0.18	3.98 ± 0.20	0.65 ± 0.05	0.72 ± 0.05	2.06 ± 0.08	3.43 ± 0.13	2.95 ± 0.22
11	Iproniasid	14	5.05 ± 0.41	3.83 ± 0.11	0.65 ± 0.06	† 0.53 ± 0.05	2.18 ± 0.14	3.37 ± 0.20	‡ 4.26 ± 0.36
	Phenelzine	5	4.91 ± 0.56	4.10 ± 0.17	0.72 ± 0.06	† 0.49 ± 0.05	* 2.47 ± 0.13	3.68 ± 0.19	‡ 5.30 ± 0.36
	Control	0	5.58 ± 0.91	4.39 ± 0.15	0.82 ± 0.03	0.76 ± 0.03	2.49 ± 0.03	4.07 ± 0.06	3.31 ± 0.15
12	Iproniasid	20	5.36 ± 0.42	4.40 ± 0.08	0.86 ± 0.04	‡ 0.65 ± 0.03	‡ 2.84 ± 0.07	4.33 ± 0.12	† 4.41 ± 0.18
	Isoniasid	20	4.86 ± 0.33	4.37 ± 0.28	0.75 ± 0.06	‡ 0.59 ± 0.03	‡ 2.92 ± 0.05	4.16 ± 0.10	† 4.96 ± 0.26
	Control	0	5.21 ± 0.69	4.02 ± 0.11	0.78 ± 0.05	0.75 ± 0.05	2.44 ± 0.07	3.97 ± 0.11	3.32 ± 0.18
13	Iproniazid	40	5.11 ± 0.39	4.03 ± 0.17	0.71 ± 0.03	‡ 0.48 ± 0.02	‡ 3.02 ± 0.10	* 4.22 ± 0.13	‡ 6.32 ± 0.21
	Isoni[illegible]	40	4.90 ± 0.63	3.96 ± 0.09	0.78 ± 0.03	‡ 0.46 ± 0.03	‡ 2.90 ± 0.10	4.10 ± 0.13	‡ 6.55 ± 0.49
	Control	0	4.11 ± 0.31	4.13 ± 0.32	0.96 ± 0.04	0.85 ± 0.05	2.47 ± 0.08	4.29 ± 0.08	2.98 ± 0.21
14	Pheniprazine	1	4.95 ± 0.42	4.03 ± 0.14	0.82 ± 0.05	0.69 ± 0.04	† 3.04 ± 0.07	4.56 ± 0.12	† 4.51 ± 0.32
	Tranylcypromine	2	5.11 ± 0.90	4.02 ± 0.26	0.82 ± 0.04	0.75 ± 0.04	† 2.83 ± 0.09	4.40 ± 0.04	† 3.85 ± 0.27
	Control	0	4.57 ± 0.34	4.03 ± 0.09	0.89 ± 0.04	0.88 ± 0.03	2.61 ± 0.07	4.37 ± 0.09	3.00 ± 0.14
15	Harmine	20	5.09 ± 0.48	* 3.57 ± 0.15	0.92 ± 0.03	0.85 ± 0.05	2.51 ± 0.13	4.28 ± 0.15	2.99 ± 0.19
	Cocaine	20	4.49 ± 0.23	* 3.64 ± 0.09	0.94 ± 0.06	0.91 ± 0.05	2.29 ± 0.07	4.14 ± 0.13	* 2.59 ± 0.15
	Control	0	5.42 ± 0.99	3.86 ± 0.17	0.68 ± 0.03	0.68 ± 0.03	2.64 ± 0.09	3.97 ± 0.08	3.95 ± 0.25
16	Cocaine	30	5.68 ± 0.57	4.09 ± 0.10	0.69 ± 0.06	0.75 ± 0.03	2.39 ± 0.15	3.94 ± 0.22	3.28 ± 0.16

Significance of differences from control: * $0.05 > P > 0.01$ † $0.01 > P > 0.001$ ‡ $0.001 > P$

were produced by doses of 15 to 40 mg/kg.

The levels of adenine nucleotides, inorganic phosphate and phosphocreatine observed 3 hours after treatment with these drugs are shown in Table 10. There was no change in the levels of inorganic phosphate or AMP, and with the exception of cocaine and harmine (series 15), which caused a decrease, there was no change in the phosphocreatine level. An increase in the total adenine nucleotide content (AMP + ADP + ATP) was observed only after 40 mg/kg of iproniazid.

Although the ATP/ADP ratio was lower after 20 mg/kg of cocaine (series 15), the ADP and ATP levels did not change either after this dose or after 30 mg/kg (series 16). Harmine (20 mg/kg; series 15) produced no changes in the ATP/ADP ratio or in the levels of ADP and ATP.

However, after all of the other drugs, there was a rise in the ATP/ADP ratio but the increase in the ATP level was only significant after phenelzine (5 mg/kg; series 11), iproniazid and isoniazid (20 and 40 mg/kg each; series 12 & 13), and pheniprazine and tranylcypromine (1 and 2 mg/kg respectively; series 14). The decrease

Table 11

In Vivo effect of Harmine (½ hour after treatment) and Cocaine (1½ hours after treatment) on the Adenine Nucleotide, Inorganic Phosphate and Phosphocreatine Concentrations in the Rat Brain.

All values are the mean of observations on 9 rats ± S.E. of mean.

Series	Treatment	Dose (mg/kg)	Time After Treatment (hr.)	Concentration in μmoles per g Frozen Tissue						Ratio
				Inorganic Phosphate	Phospho-creatine	AMP	ADP	ATP	AMP + ADP + ATP	$\frac{ATP}{ADP}$
17	Control	0	½	5.98 ± 0.95	4.60 ± 0.18	0.94 ± 0.07	0.75 ± 0.05	2.48 ± 0.14	4.17 ± 0.14	3.40 ± 0.32
	Harmine	10	½	5.20 ± 0.47	4.71 ± 0.15	0.82 ± 0.06	0.72 ± 0.06	2.50 ± 0.06	4.05 ± 0.12	3.65 ± 0.31
	Harmine	40	½	4.63 ± 0.44	4.90 ± 0.18	0.77 ± 0.06	0.62 ± 0.04	2.70 ± 0.13	4.09 ± 0.17	4.52 ± 0.36
18	Control	0	1½	5.78 ± 0.63	2.57 ± 0.08	0.89 ± 0.06	0.74 ± 0.02	2.25 ± 0.03	3.89 ± 0.08	3.03 ± 0.09
	Cocaine	15	1½	5.50 ± 0.70	2.52 ± 0.08	0.85 ± 0.05	0.69 ± 0.02	†2.51 ± 0.08	4.04 ± 0.11	†3.66 ± 0.13
	Cocaine	30	1½	5.22 ± 0.30	2.51 ± 0.07	0.83 ± 0.03	0.68 ± 0.02	†2.54 ± 0.06	4.06 ± 0.05	†3.75 ± 0.19

Significance of differences from control: †0.01 > P > 0.001

in the ADP level was significant after all of these drugs except pheniprazine and tranylcypromine. In these cases where an increase in the ATP/ADP ratio was observed, the drug-treated animals were generally more alert than the controls during the time immediately before they were killed. It is of particular interest to note that isoniazid and iproniazid produced similar effects on brain adenine nucleotide levels, since both compounds, in the higher dose, kept the animals awake to the same extent (Table 9). Since isoniazid is a very weak inhibitor of monoamine oxidase (Zeller et al., 1952), and iproniazid is relatively much more potent in this respect, there does not seem to be a relationship between the inhibition of this enzyme and the ability of the drugs to produce signs of central nervous system stimulation and to increase the ratio of ATP/ADP in the rat brain.

The effects of harmine ½ hour after treatment when the tremor was distinct are shown in Table 11 (series 17). Neither 10 mg/kg, which produced mild tremor, nor 40 mg/kg, which produced marked tremor, caused any significant change in the levels of inorganic

Table 12

In Vivo effect of Pheniprazine (2 mg/kg) and Tranylcypromine (8 mg/kg) on the Adenine Nucleotide, Inorganic Phosphate and Phosphocreatine Concentrations in the Rat Brain at Various Times after Treatment.

All values are the mean of observations on 9 rats ± S.E.M.

Series	Treatment	Time After Treatment (hr)	Concentration in μmoles per g Frozen Tissue						Ratio
			Inorganic Phosphate	Phospho-creatine	AMP	ADP	ATP	AMP + ADP + ATP	ATP / ADP
19	Control	½	5.35 ± 0.78	3.72 ± 0.13	0.82 ± 0.06	0.76 ± 0.05	2.41 ± 0.06	3.92 ± 0.12	3.28 ± 0.24
	Pheniprazine	½	4.24 ± 0.29	3.96 ± 0.13	0.72 ± 0.04	0.63 ± 0.04	2.57 ± 0.13	3.93 ± 0.16	* 4.20 ± 0.34
	Tranylcypromine	½	4.32 ± 0.39	3.66 ± 0.12	0.77 ± 0.05	0.63 ± 0.06	2.48 ± 0.11	3.88 ± 0.15	* 4.15 ± 0.37
20	Control	3	4.52 ± 0.25	4.45 ± 0.15	0.77 ± 0.05	0.80 ± 0.03	2.59 ± 0.06	4.19 ± 0.06	3.28 ± 0.18
	Pheniprazine	3	6.04 ± 0.94	4.77 ± 0.18	† 0.60 ± 0.05	† 0.62 ± 0.05	‡ 3.13 ± 0.08	4.36 ± 0.12	† 5.26 ± 0.49
	Tranylcypromine	3	4.09 ± 0.31	4.55 ± 0.15	0.74 ± 0.05	† 0.68 ± 0.03	‡ 2.82 ± 0.06	4.24 ± 0.06	† 4.33 ± 0.29
21	Control	6	5.02 ± 0.72	3.61 ± 0.10	0.82 ± 0.06	0.82 ± 0.06	2.33 ± 0.09	3.98 ± 0.15	2.93 ± 0.23
	Pheniprazine	6	5.71 ± 1.10	3.74 ± 0.36	0.66 ± 0.07	† 0.64 ± 0.04	* 2.70 ± 0.11	3.96 ± 0.19	* 4.30 ± 0.27
	Tranylcypromine	6	5.11 ± 0.31	3.42 ± 0.10	0.77 ± 0.08	† 0.66 ± 0.04	* 2.68 ± 0.15	4.12 ± 0.10	* 4.20 ± 0.39
22	Control	12	4.18 ± 0.18	4.15 ± 0.17	0.77 ± 0.08	0.81 ± 0.05	2.64 ± 0.07	4.23 ± 0.11	3.38 ± 0.29
	Pheniprazine	12	5.53 ± 1.02	4.11 ± 0.14	0.81 ± 0.03	0.82 ± 0.03	2.56 ± 0.11	4.19 ± 0.13	3.14 ± 0.13
	Tranylcypromine	12	4.79 ± 0.39	4.07 ± 0.21	0.66 ± 0.02	0.72 ± 0.05	2.80 ± 0.07	4.18 ± 0.07	4.06 ± 0.32

Significance of differences from control at the same time interval: * $0.05 > P > 0.01$ † $0.01 > P > 0.001$ ‡ $P > 0.001$

phosphate, phosphocreatine, AMP, ADP, ATP or in the ATP/ADP ratio. Thus, although harmine is a potent monoamine oxidase inhibitor, it neither produces characteristic signs of central nervous system stimulation nor affects brain phosphate metabolism in the rat.

The effect of cocaine was studied 1½ hour after treatment because, at this time, signs of central nervous system stimulation were obvious. After both 15 and 30 mg/kg there were significant increases in the ATP/ADP ratio and in the ATP level. Although there was a small decrease in the ADP level, neither this nor any of the other values changed significantly.

An attempt was also made to find out if an increase in the ATP/ADP ratio could be demonstrated at different times after treatment with pheniprazine and tranylcypromine when behavioural signs of central nervous system stimulation were observed. From Table 12 it can be seen that there was no change in the levels or inorganic phosphate, phosphocreatine, or in the total adenine nucleotide content (AMP + ADP + ATP) at any of the time intervals. The AMP level was decreased 3 hours after pheniprazine. However, ½, 3 and 6 hours after treatment with both drugs, the ATP/ADP ratio was

significantly increased, but after 12 hours it was back to the control level. The rise in the ATP level and the fall in the ADP level was only significant 3 and 6 hours after treatment. There seems, therefore, to be an approximate relationship between the central stimulant action of these two drugs (see page 98) and the increase in the ATP/ADP ratio.

Antidepressives Resembling Phenothiazine in Chemical Structure and other Related Compounds.

Gross Observations:

When compared with the compounds previously studied, the behavioural signs of central nervous system stimulation produced by the drugs in this group were less obvious. Those rats treated with imipramine were little different in appearance from the controls in so much as they seemed to sleep about the same amount of time while they were in the small cages. They were, however, considerably more responsive to external stimuli. Noises such as that produced by the turning on or off of a switch startled them. If they were touched with a pencil, they seemed frightened and tried to bite it. The rats treated with amitriptyline and

Table 13

In Vivo effect of Imipramine, Amitriptyline, Orphenadrine and Diphenhydramine on the Adenine Nucleotide, Inorganic Phosphate and Phosphocreatine Concentrations in the Rat Brain.

All values are the means of observations on 9 rats ± S.E. of mean obtained 3 hours after injection of the drug or control solutions.

Series	Treatment	Dose (mg/kg)	Concentration in μmoles per g Frozen Tissue						Ratio
			Inorganic Phosphate	Phospho-creatine	AMP	ADP	ATP	AMP + ADP + ATP	$\frac{ATP}{ADP}$
23	Control	0	4.40 ± 0.35	4.13 ± 0.13	0.87 ± 0.03	0.87 ± 0.03	2.42 ± 0.04	4.16 ± 0.06	2.81 ± 0.09
	Amitriptyline	25	4.46 ± 0.24	3.87 ± 0.11	*0.70 ± 0.07	‡0.51 ± 0.05	*2.83 ± 0.11	4.04 ± 0.20	†6.29 ± 0.95
	Imipramine	25	5.32 ± 0,63	4.18 ± 0.07	0.86 ± 0.03	0.83 ± 0.07	2.58 ± 0.09	4.27 ± 0.10	3.32 ± 0.33
24	Control	0	5.32 ± 0.32	3.96 ± 0.17	0.80 ± 0.02	0.79 ± 0.02	2.46 ± 0.05	4.05 ± 0.06	3.11 ± 0.07
	Imipramine	50	5.05 ± 0.43	4.22 ± 0.22	0.74 ± 0.03	0.77 ± 0.05	†2.59 ± 0.06	3.92 ± 0.12	3.46 ± 0.21
	Orphenadrine	15	5.05 ± 0.41	4.24 ± 0.19	0.75 ± 0.03	0.69 ± 0.03	†2.74 ± 0.07	4.16 ± 0.07	*4.04 ± 0.23
25	Control	0	4.23 ± 0.12	4.07 ± 0.16	0.85 ± 0.07	0.90 ± 0.03	2.44 ± 0.07	4.19 ± 0.09	2.73 ± 0.11
	Imipramine	50	5.78 ± 0.79	3.86 ± 0.21	0.81 ± 0.04	0.83 ± 0.04	†2.66 ± 0.07	4.31 ± 0.08	‡3.25 ± 0.21
	Orphenadrine	30	6.08 ± 1.16	3.80 ± 0.17	0.79 ± 0.03	*0.76 ± 0.04	†2.82 ± 0.02	4.36 ± 0.06	‡3.82 ± 0.22
§ 16	Control	0	5.43 ± 0.99	3.86 ± 0.17	0.68 ± 0.03	0.68 ± 0.03	2.64 ± 0.09	3.97 ± 0.08	3.95 ± 0.25
	Diphenhydramine	30	4.76 ± 0.31	3.98 ± 0.15	0.72 ± 0.06	†0.86 ± 0.03	2.55 ± 0.06	4.14 ± 0.09	3.01 ± 0.16

Significance of differences from control: *0.05 > P > 0.01 †0.01 > P > 0.001 ‡0.001 > P

§ The experiments on diphenhydramine were done in conjunction with cocaine (see Table 10) but for convenience in comparing the effects of diphenhydramine with the closely related compound, orphenadrine, the results are tabulated separately.

orphenadrine seemed to sleep less than the controls but did not show signs of hypermotility. On the other hand, after treatment with diphenhydramine, which only lacks the ortho-methyl group of orphenadrine, the rats seemed to sleep more than the controls. This was not surprising since one of the common side effects of the antihistaminic properties of diphenhydramine is sedation (Goodman & Gilman, 1955).

Effects on Brain Levels of Adenine Nucleotides, Inorganic Phosphate and Phosphocreatine.

The effects of the drugs in this group on the brain levels of adenine nucleotides, inorganic phosphate and phosphocreatine 3 hours after treatment are shown in Table 13. None of these drugs caused any significant change in the brain levels of inorganic phosphate, or phosphocreatine, or in the total adenine nucleotide content (AMP + ADP + ATP).

While the increases in the ATP level and the ATP/ADP ratio, and the decreases in the ADP and AMP levels were all significant after treatment with 25 mg/kg of amitriptyline, this dose of imipramine produced no significant effects (series 23). After treatment with

the larger dose of 50 mg/kg of imipramine (series 24), there was a significant increase in the ATP level, but neither the ADP level nor the ratio of ATP/ADP were changed significantly. When this same dose was repeated in series 25, both the rise in the ATP level and the ratio of ATP/ADP were significant. That the increase in the ratio of ATP/ADP was not significant in series 24, but was significant in series 25 is probably due to the different coefficients of variation in the two series (series 24 = 17.7 %; series 25 = 12.9 %). After 15 mg/kg of orphenadrine, the ATP level and the ratio of ATP/ADP increased significantly, but the ADP level was not affected (series 24). After 30 mg/kg, however, the decrease in the ADP level and increases in the ATP level and the ratio of ATP/ADP were all significant (series 25). It is of interest to note that the same dose (30 mg/kg) of diphenhydramine tended to produce the opposite effects; there was a significant increase in the ADP level and the ATP level and the ratio of ATP/ADP tended to decrease (series 16). The tendency of the chemically closely related compounds, orphenadrine

and diphenhydramine, to produce opposite effects on the ADP and ATP levels of the brain is of particular interest, since these two drugs also seem to produce opposite actions on the central nervous system.

While amitriptyline and orphenadrine produced similar changes in the adenine nucleotide levels of the brain to the central nervous system stimulants of the previous groups, imipramine appeared to be somewhat different. That is, after the rather high dose of 50 mg/kg of imipramine, the ATP level increased but the ADP level did not decrease. Since this dose of imipramine was close to the dose of 75 mg/kg which was found to be toxic, the increase in the ATP level may be non-specific and unrelated to the antidepressive action of the drug. In any case, imipramine does not produce typical signs of central nervous system stimulation in the rat.

SUMMARY OF RESULTS

In summary, it was found with these three groups of drugs that when signs of central nervous system stimulation were observed (alertness, tendency to stay awake and in some cases hypermotility), the brain ATP level was significantly higher and the ADP level significantly lower. This effect was not observed when the drugs did not produce signs of central nervous system stimulation (e.g. 0.45 mg/kg l-amphetamine; 0.90 & 1.80 mg/kg l-ephedrine; 3.60 mg/kg l-phendimetrazine; 10, 20 & 30 mg/kg harmine; 20 & 30 mg/kg cocaine 3 hours after injection; 2 mg/kg pheniprazine & 8 mg/kg tranylcypromine 12 hours after injection; 30 mg/kg diphenhydramine; imipramine doubtful). There seems, therefore, to be some relationship between the central nervous system stimulation produced by a number of drugs and an increase in the brain ATP level and decrease in the brain ADP level in the rat.

DISCUSSION

Table 14

Levels of Inorganic Phosphate, Phosphocreatine and Adenine Nucleotides in the Rat Brain *in vivo*: Comparison of Control Values Found in the Present Work with Some Values Found by Other Workers.

Reference	Method of Killing	umoles per g frozen brain ± S.D.						
		Inorganic Phosphate	Phospho-creatine	AMP	ADP	ATP	AMP + ADP + ATP	Ratio ATP/ADP
Present Work	Intact Rat Frozen	5.22 ±2.04	3.80 ±0.64	0.81 ±0.17	0.83 ±0.17	2.29 ±0.29	3.93 ±0.35	2.89 ±0.23
1	Intact Rat Frozen	4.14	2.46	0.26	0.57	1.77	2.60	3.10
2	Intact Rat Frozen	3.95	2.49	0.25	0.60	1.76	2.61	2.93
3	Intact Rat Frozen		3.21	0.46	0.99	2.58	4.03	2.61
4	Intact Rat Frozen							
	(a) rats 1 day old		2.99	0.11	0.14	1.97	2.22	15.02
	(b) rats 5 days old		2.63	0.14	0.20	2.10	2.44	13.55
	(c) rats 10 days old		2.84	0.16	0.34	2.10	2.60	7.68
	(d) rats 21 days old		2.18	0.17	0.39	1.99	2.55	5.40
5	(a) Intact Rat Frozen		2.02	0.42	0.66	1.22	2.30	1.85
	(b) Rat Decapitated, Head Immediately Frozen		0.67	1.00	0.86	0.58	2.44	0.67
6	(a) Intact Rat Frozen			1.16	0.45	0.17	1.78	0.38
	(b) Rat Decapitated, Head Immediately Frozen			0.03	0.18	1.41	1.62	7.83
7	(a) Intact Rat Frozen		3.45	0.08	0.24	2.05	2.37	8.54
	(b) Rat Decapitated, Head Frozen After: 15 sec		0.5	0.8		1.1		
	30 sec		0.1	1.3		0.5		

1: Doring & Gerlach, 1957; 2: Gerlach *et al.*, 1957; 3: Lin *et al.*, 1958; 4: Lolley, Balfour & Samson, 1961 5: Weiner, 1961; 6: Mandel & Harth, 1961; 7: Minard & Davis, 1962. The methods of assay were as follows: 1 & 2, paperchromatographic; 4, 6 & 7, column chromatographic; 5, enzymic (Kalckar, 1947b).

DISCUSSION

The estimation of phosphate esters in the brain *in vivo* presents certain difficulties. In particular, phosphocreatine and ATP, and to a lesser extent ADP, are subject to rapid enzymic hydrolysis after death and even in deproteinized solution they are quite unstable, especially at extremes of pH. These factors must be born in mind in assessing the results of any estimations.

The average control levels of inorganic phosphate, phosphocreatine (224 observations) and adenine nucleotides (263 observations) are shown in Table 14. A certain amount of variation between the control values obtained in the different series of experiments is evident from the previous tables of the results (see Tables 6, 7, 8, 10, 11, 12 & 13). With the exception of the inorganic phosphate level, this variation was not excessive in view of the long period of time over which the results were obtained, the possible variation between animals and other uncontrollable factors. In the case of inorganic phosphate, the large variation appears to be due to the very high levels found in certain early

experiments. These probably resulted from contamination of the extracts with small chips of bone.

From Table 14, it can be seen that the results obtained by different workers show considerable variation. In general, however, they have obtained lower values, particularly of AMP, ADP and ATP. With the exception of the values found by Lin and his associates (1958; Table 14, reference 3), the total adenine nucleotide content (AMP + ADP + ATP) is, therefore, considerably lower than that found in the present work. Although it is difficult to account for this discrepancy, one immediately thinks of the possible hydrolysis of ATP to ADP and ADP to AMP. This would result in a redistribution of the mono-, di- and tri-phosphates but would not affect the total adenine nucleotide content. Moreover, hydrolysis of ATP would probably lead to an increase in the ADP level and a decrease in the ATP/ADP ratio. Yet those workers who have obtained a markedly higher ATP/ADP ratio (Table 14, references 4, 6 & 7) also reported lower ATP levels than those found in the present work. The data of Lolley and his associates (1961; Table 14, reference 4)

indicate that the total adenine nucleotide content increases with the age of the rat. If such a trend continued, one might expect a higher total in the present work where rats about six weeks old were used. However, Minard and Davis (1962; Table 14, reference 7) found a lower total adenine nucleotide content in adult rats weighing from 250 to 400 g. The possibility that the present high total adenine nucleotide content is due to a difference between enzymic and chromatographic methods of assay seems unlikely since Weiner (1961; Table 14, reference 5) found a lower value, despite the fact that he also used Kalckar's (1947b) enzymic method of assay. Moreover, the adenine nucleotide levels found by Lin and his associates, using a chromatographic method, (1958; Table 14, reference 3) agree well with the present study. The deamination of AMP to form inosinemonophosphate could lead to a decrease in the total adenine nucleotide content. This seems unlikely, since under conditions where a large amount of hydrolysis of ATP seems to have occurred (i.e. decapitation of the rat and extraction of the brain without freezing; Table 4, opposite page 82), a high AMP level (2.09 µmoles/g) was

found and the total adenine nucleotide content (4.16 μmoles/g) was similar to that found using the freezing technique.

Although the relatively high total adenine nucleotide level found in the present work cannot be readily explained by hydrolysis of ATP, the comparatively high AMP and ADP levels might be explained on this basis. It is, therefore, of interest to consider three main places in the present method where the hydrolysis of energy-rich phosphates seems most likely to occur.

First, there is the possibility of enzymic breakdown during the killing of the rat and before the brain is fixed. Low ATP and high ADP and AMP levels were found in preliminary experiments when the rat was killed by decapitation and the brain removed and extracted without freezing (Table 4, opposite page 82). The technique of decapitation was also found to be difficult and these results may not only reflect the breakdown of ATP after death but may be partly attributable to the excitation of the rat before the moment of decapitation. A similar

decrease in the ATP level has been observed by other workers (Table 14, references 5 & 7) when rats were decapitated and the brain subsequently frozen. These data thus support the view that freezing the brain *in situ* minimizes the breakdown of energy-rich phosphates. Such a breakdown is known to occur during short periods of ischaemia or anoxia (Doring & Gerlach, 1957). Moreover, it is reasonable to suppose that the violent stimulation of the brain produced by decapitation would also produce breakdown of ATP (Weiner, 1962). It is thus difficult to explain the results obtained by Mandel and Harth (1961; Table 14, reference 7) which indicate that higher ATP levels are found if the rat is decapitated and the head allowed to fall into liquid nitrogen than if the whole rat is plunged into liquid nitrogen. Although there may well be some breakdown of ATP during the few seconds before the brain is solidly frozen *in situ* (Richter & Dawson, 1948), this method generally yields higher levels of ATP and phosphocreatine and lower levels of ADP and inorganic phosphate and appears to be the best available (Heald, 1960). Furthermore, emotional excitement

before plunging the rat into liquid nitrogen is known to result in a decreased brain ATP level (Le Page, 1946; Sytinsky, 1956; see page 58). In view of the observation that the ATP level falls and the ADP level rises in amphetamine-treated rats which are teased with a straw (Shapot, 1957), it was particularly important that in the present investigation the rats were not disturbed prior to immersion in liquid nitrogen. This was avoided by placing the animal in a small cage which was plunged directly into the coolant. In any case, if emotional excitement did occur during the brief period between grasping the handle of the cage and plunging it into liquid nitrogen (1 to 3 seconds), it would probably be more intense in the animals treated with drugs which kept them awake than in control animals which were often asleep at this time. Yet higher levels of ATP and lower levels of ADP were found in the animals treated with such drugs.

Secondly, some enzymic hydrolysis of labile phosphates might occur after the brain is solidly frozen and before the enzymes are denatured by the acid extraction. This possibility is particularly important

Table 15

Levels of Adenine Nucleotides in the Rat Brain *in vivo*;
Effect of Different Extraction Procedures.

All values are the mean of observations on 6 rats ± S. E. of mean.

Method of Extraction	μmoles per g of frozen brain				Ratio $\frac{ATP}{ADP}$
	AMP	ADP	ATP	AMP + ADP + ATP	
Powdered Frozen Brain Stirred for 20 Minutes with PCA at 0° C	1.15 ± 0.09	1.09 ± 0.06	1.50 ± 0.09	3.74 ± 0.13	1.39 ± 0.08
Powdered Frozen Brain Homogenized for 2 Minutes with PCA at 0° C	0.77 ± 0.05	0.79 ± 0.08	2.31 ± 0.08	3.87 ± 0.06	3.11 ± 0.45

when one considers that the enzymic dephosphorylation of ATP and phosphocreatine by brain homogenates is sufficiently rapid to permit the complete hydrolysis of the entire amounts found in the brain in a few seconds (Weiner, 1962). The danger is further increased by the relatively low temperature coefficient of activity of an adenosinetriphosphatase (Dawson & Richter, 1950; Weiner, 1961), and the relative stability of adenylate kinase in acid (Kalckar, 1947b). In preliminary experiments, in which the perchloric acid was added to the powdered frozen brain and the mixture stirred for 20 minutes at 0°C, a low ATP level and high ADP and AMP levels were obtained (Table 15). When the acid was added to the frozen powdered brain, the tissue tended to freeze into small lumps. Homogenization with a loosely fitting plastic pestle, to break up these lumps, immediately after addition of the acid to the brain resulted in a considerable increase in the ATP level and a fall in the ADP and AMP levels. Presumably these results indicate that unless the acid penetrates the powdered frozen brain quickly and completely, enzymic hydrolysis may take place during

Table 16

Levels of Adenine Nucleotides in the Rat Brain *in vivo*;
Effect of Storage of the Brain in Liquid Nitrogen.

All values are means of observations on a number of rats ± S. E. of mean.

Time Stored	Number of Observations	µmoles per g of frozen brain				Ratio $\frac{ATP}{ADP}$
		AMP	ADP	ATP	AMP + ADP + ATP	
*	6	0.77 ± 0.05	0.79 ± 0.08	2.31 ± 0.08	3.87 ± 0.06	3.11 ± 0.45
24 hours	6	0.79 ± 0.06	0.69 ± 0.06	2.24 ± 0.05	3.72 ± 0.08	3.39 ± 0.53
48 hours	6	0.81 ± 0.02	0.73 ± 0.05	2.27 ± 0.07	3.81 ± 0.07	3.24 ± 0.37
14 days	2	0.75 ± 0.21	0.71 ± 0.11	2.36 ± 0.05	3.82 ± 0.27	3.40 ± 0.62

* Extractions and assays performed as soon as possible after the rats were killed.

the extraction. Although Weiner (1961) reported that homogenization of the tissue with a glass homogenizer did not improve the recoveries of adenine nucleotides and phosphocreatine, his values, not only for these substances but also for AMP and ADP (see Table 14, reference 6) are lower than those obtained in the present work, despite the fact that he also used Kalckar's (1947b) enzymic method of assay. In other preliminary experiments, the brain was extracted and assayed either as soon as possible after dissection or after storage in liquid nitrogen for 1, 2 or 14 days. Since similar levels of adenine nucleotides, inorganic phosphate and phosphocreatine were found in all cases, no hydrolysis seems to have occurred during storage of the brain under these conditions (Table 16). In practice the brain was only stored for up to 24 hours.

The third possibility is that non-enzymic hydrolysis might occur either in the acid extract or in the portion which was buffered to pH 6.1 for the assay of adenine nucleotides. To minimize this danger, the assays were begun as soon as possible after the extraction was completed.

There is, therefore, little reason to think that there was any significant breakdown of ATP or phosphocreatine during the present experiments. In any case, the procedure was the same for both drug-treated and control rats so that any breakdown of energy-rich phosphates might be expected to be the same in both cases and, provided it is not very large, should not invalidate a comparison. It is of course conceivable that the presence in the brain of the drugs being studied might diminish the breakdown of ATP by an adenosinetriphosphatase during the extraction. Such an effect is, however, unlikely since 10^{-2} M amphetamine (i.e. 100 to 1000 times the concentration attained *in vivo*) only slightly depresses the activity of this enzyme *in vitro* (Lewis & Pollock, unpublished). Finally, the use throughout of random statistical designs should minimize any bias which might occur as a result of variations between animals or differences in experimental technique during the time required to complete a series of experiments.

Within the limitations of the methods employed, the results of this investigation indicate that the

brain level of ATP is increased and the brain level of ADP decreased in rats when behavioural signs of central nervous system stimulation are elicited by drugs. These changes are reflected in an increased ATP/ADP ratio. This could be due either to a decreased utilization or to an increased synthesis of ATP. If signs of excitement and fear are produced in experimental animals by the application of external mechanical or electrical stimulation (see page 58), it has been shown that the ATP level of the brain falls. Since under such conditions there is probably greater nervous activity in the brain, the fall in ATP is generally believed to reflect increased utilization of ATP to support the greater activity. The electrical activity of the brain is known to increase after the administration of amphetamine-like central nervous system stimulants (see pages 17 to 20) and an increased utilization of ATP might also be expected in this case. Yet an increase in the ATP level was observed. This would seem, therefore, to suggest that as well as an increased utilization of ATP, there is an even greater increase in its synthesis. It is conceivable, however,

that the depression of a modulating area of the central nervous system (e.g. the recruiting response of the diffuse thalamocortical system) could lead to increased activity of higher centres (e.g. cerebral cortex) and behavioural signs of stimulation. In the area where activity is depressed, one might expect a decreased utilization of energy, while in the area where activity is increased a greater utilization of energy might occur. Depending upon the balance between these two effects, there might be either a decrease or an increase in the ATP content of the brain as a whole. ATP seems to be fairly evenly distributed in the brain in so far as gross areas have been examined (cerebral cortex, cerebellum, medulla and thalamus-hypothalamus; Grenell et al., 1955). Since the cerebral cortex represents a large proportion by weight of the whole brain, a decreased utilization of ATP in the rest of the brain would have to be large to cause a rise in the ATP level in the brain as a whole.

The phosphocreatine levels did not change after the administration of the central nervous system stimulants used in this study, despite the increase in the ATP level.

This may be surprising if it is assumed that an equilibrium exists between the ADP-ATP and the phosphocreatine-creatine systems and the two systems are homogeneously distributed in the brain. It is of interest to note that during anaesthesia an increase in the phosphocreatine content has been found when there was no change in the ATP level. This is directly opposite to the present observations. Although technical difficulties are involved, such experimental findings may indicate that a theoretical equilibrium does not exist in a system as complicated and heterogeneous as the whole brain.

Although such arguments favour the idea that the central nervous system stimulation produced by a number of drugs is associated with a net increase in the synthesis of ATP in the brain, this has not been proved by the results of the present investigation. The increase in the ATP/ADP ratio only indicates an alteration in the balance between energy utilization and production and gives no information as to how the change is brought about. The literature on these drugs does not throw much light on this question. A tendency

for low concentrations (10^{-3} - 10^{-4} M) of amphetamine to increase the content of ATP in rat cerebral cortex slices has been reported (Lisovskaya & Livanova, 1959). Central nervous system stimulant drugs appear to produce little or no effect on brain respiration either *in vivo* or when they are added to the tissue *in vitro* (see pages 56 to 57). The *in vitro* oxygen consumption of brain removed from animals treated with methylamphetamine (Utena, Ezoe, Kato & Hada, 1959), amphetamine and a number of monoamine oxidase inhibitors has been observed to be higher than that of brain removed from control animals (Greig, Walk & Gibbons, 1959). This effect was not produced in liver (Greig *et al.*, 1959). The intensity of anaerobic glycolysis is increased by methylamphetamine (Palladin, 1952). While both increased oxidation and glycolysis could result in an increased synthesis of ATP, the oxidative pathway is more efficient. Moreover, although glycolysis can proceed at a higher rate than oxidation *in vitro* (McIlwain, 1959 and references cited), the precise role of the anaerobic pathway *in vivo* has not been established. In any case, the present lack of evidence that the

administration of these drugs causes significant changes in either oxidation or glycolysis makes it difficult to envisage a way in which the rate of synthesis of ATP in the brain might be increased.

If it is assumed that central nervous system stimulant drugs make more ATP available for brain functions, the question arises as to what particular processes may be affected to bring about an increase in brain activity. Most of the current theories on the mode of action of these drugs consider that they modify in some way central neurohumoral transmission. It is therefore of interest to consider whether an increased ATP level in the brain might affect such processes. There is evidence that ATP may be used for the synthesis of both noradrenaline (Kirshner, 1959, 1960), and acetylcholine (McIlwain, 1959 and references cited), and a greater amount of ATP might allow the synthesis of these compounds to proceed at a higher rate. ATP may also be involved in the binding of catecholamines in the adrenal medulla (Hillarp, 1960 and references cited). If such a mechanism operates in the brain, a change in the amount of ATP available might affect the

amount of transmitter released to act on the appropriate receptor. The inactivation of catecholamines by the enzyme catechol-O-methyltransferase may also require ATP (Axelrod et al., 1959) and this could influence the concentration of transmitter at the receptor sites. If any one of these actions does occur, then some direct or inverse relationship between the levels of transmitters and ATP might be expected. No such relationship was revealed in the present experiments. Thus amphetamine lowers the noradrenaline content of the brain and increases the ATP content. The levels of noradrenaline and 5-hydroxytryptamine are increased by many monoamine oxidase inhibitors (e.g. iproniazid, phenelzine, pheniprazine, tranylcypromine & harmine), but the ATP level is not always increased (e.g. harmine). Other drugs which apparently have little or no effect on the levels of these amines did increase the brain ATP levels (e.g. isoniazid, amitriptyline & orphenadrine). Moreover, the time course of the increase in brain amine levels and ATP produced by pheniprazine and tranylcypromine is different. The ATP level is high 3 and 6 hours after treatment with these drugs but is normal 12 hours

after treatment. In contrast, the noradrenaline level rises to a maximum about 5 hours after treatment and then falls very slowly and is still well above normal 24 hours after treatment (Crout, Creveling & Udenfriend, 1961). Indeed, 2 days after the oral administration of 5 mg/kg of tranylcypromine, the noradrenaline level of the rat brain is about 50 per cent above normal and after 7 days it is still 15 per cent above normal (Green & Erickson, 1960). Similarly, the brain level of 5-hydroxytryptamine rises to a maximum 6 hours after the administration of pheniprazine, remains high for 15 hours, and is still twice the normal level after 48 hours (Prockop, Shore & Brodie, 1959). Although the brain acetylcholine levels do not apparently change after the administration of iproniazid (Giarman & Pepeu, 1962), the effects of the other drugs used in this investigation on brain acetylcholine levels are unknown. No justifiable comparison can be made between drug-induced changes in brain ATP and acetylcholine levels. The present experiments do not, therefore, show any simple relationship between the ability of a drug to alter the levels of ATP and the levels of central transmitters so far investigated by

others. Neither do they exclude such a possibility. A relationship with the functions of acetylcholine or other transmitters, the levels of which have not been studied after the administration of central nervous system stimulant drugs, may exist. Moreover, if an increased ATP level facilitated both the binding or synthesis and the inactivation of noradrenaline, then, depending upon the balance between these two mechanisms, the level of the transmitter could increase, decrease or not change. Such an effect does, however, seem unlikely. For example, the chemically and pharmacologically similar drugs, amphetamine and pheniprazine, both increase the brain ATP level, yet produce opposite effects on the level of noradrenaline. Thus in one case the extra ATP would have to be channeled towards increased inactivation and in the other case increased synthesis or binding of this transmitter.

In peripheral nerves, it seems likely that the energy required for the maintenance of ionic gradients across the nerve cell membrane may be supplied by ATP (Hodgkin, 1958; Nachmansohn, 1959). In the brain, the high turnover rate of ATP and phosphocreatine compared

with, for example, the rate of acetylcholine synthesis, indicates that the largest portion of the energy made available by energy-rich phosphates is used for other functions, of which the maintenance of ionic balances against an electrochemical gradient is probably prominent (Dawson & Richter, 1950; Richter, 1952; Heald, 1960; McIlwain, 1959, 1962). Certain evidence from studies of peripheral nerve transmission make it difficult, however, to accept that an increase in the available energy would allow a greater rate of nervous activity by maintaining ionic gradients in a more favourable state. For example, cyanide prevents the extrusion of sodium ions from the squid giant axon but the fibre is still able to conduct impulses for many hours (Hodgkin & Keynes, 1955). One may speculate that because of its extreme complexity and the many feed-back circuits, the rate of impulse transmission in the neurones of the brain is very high compared to peripheral neurones. Thus a greater expenditure of energy may be required to maintain ionic gradients. Under such circumstances it is conceivable that an increased availability of energy might facilitate

nervous activity.

It has so far been assumed that the increased ATP level of the brain found after the administration of a number of central nervous system stimulant drugs is the result of a primary effect of the drugs. This may not be true. If one considers a sequence of reactions linking the depolarization and repolarization of a nerve membrane to the conversion of ADP to ATP and of ATP to ADP (see figure 14 of Heald, page 120, 1960), it seems probable that increased nervous activity results in a speeding up of this exchange. If, for example, a central nervous system stimulant drug caused increased nervous activity in the brain in some other way (e.g. amphetamine may have a direct central sympathomimetic action and cocaine may act by releasing catecholamines in the brain; van Rossum, van der Shoot & Hurkmans, 1962), this could result in a greater exchange between ATP and ADP. As a result of such a feed-back mechanism, a new dynamic equilibrium might be set up between energy utilization and production and so the ATP/ADP ratio could change.

SUMMARY AND CONCLUSIONS

The effects of three groups of central nervous system stimulants (amphetamine-like, monoamine oxidase inhibitors, and antidepressives resembling phenothiazine in chemical structure) and some related compounds on the levels of adenine nucleotides, inorganic phosphate and phosphocreatine in the rat brain have been investigated. In all cases where the drugs produced signs of central nervous system stimulation (e.g. appropriate doses of: d-methylamphetamine, d-amphetamine, dl-phenmetrazine, l-amphetamine, l-ephedrine, d-phendimetrazine, phenelzine, iproniazid, isoniazid, tranylcypromine, pheniprazine, cocaine, amitriptyline and orphenadrine) the level of ATP was increased and the level of ADP decreased in the brain. Where no obvious signs of central nervous system stimulation were observed, there was no effect on the brain levels of ADP or ATP. This was shown with low doses of l-amphetamine, l-ephedrine and d-phendimetrazine, l-phendimetrazine, harmine, cocaine (3 hours after injection), and pheniprazine and tranylcypromine (12 hours after treatment). Diphenhydramine, which produced sedation instead of

stimulation, tended to cause the opposite effect on the brain ADP and ATP levels.

The increase in the ATP level and the decrease in the ADP level may be due either to an increased synthesis or to a decreased utilization of ATP. Since there is probably an increase in the utilization of ATP to support the increased electrical activity of the brain, it is believed that the increased level of ATP may be due to a net increase in its synthesis. How this might be brought about is unknown.

No relationship has been demonstrated between the changes in the levels of the hypothetical central transmitters reported by other workers to be produced by these drugs and the increase in the ATP levels observed in this investigation. The possibility that the increased ATP level may play a role in the maintenance of ionic gradients in the neurones of the central nervous system, and thus facilitate nervous transmission, is discussed.

From the relatively close relationship found between signs of central nervous system stimulation produced in the rat by the drugs investigated and the increased ATP level and decreased ADP level in the brain, it is concluded that this may be an important factor in their action. Whether this is a primary or secondary effect of the drugs remains a matter for further investigation.

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APPENDICIES

APPENDIX I

Preparation of the Reagents Used.

(In all cases the water used was distilled or deionized).

Adenosine Monophosphate (AMP), 5 µmoles/ml: 0.0194 g Sigma AMP·Na·H_2O dissolved in about 7 ml ice-cold water, pH adjusted to 7 with 1 N NaOH, volume made up to 10 ml with ice-cold water, and 1 ml aliquots frozen and stored at -15°C.

Adenosine Diphosphate (ADP), 5 µmoles/ml: 0.0261 g Sigma ADP·2Na·$3H_2O$ dissolved in about 7 ml ice-cold water and made up as described for AMP.

Adenosine Triphosphate (ATP), 5 µmoles/ml: 0.0303 g Sigma ATP·2Na·$3H_2O$ dissolved in about 7 ml of ice-cold water and made up as described for AMP.

Ammonium Acetate, 1 M, pH 8: 19.27 g B.D.H. "Analar" NH_4COOCH_3 dissolved in about 225 ml water, titrated to pH 8 by adding 1 N NaOH, and volume adjusted to 250 ml with water.

Ammonium Sulphate, Saturated Solution: 75.4 g B.D.H. "Analar" $(NH_4)_2SO_4$ added to 100 ml of water, warmed to about 25°C and stirred to dissolve, cooled to 20°C and filtered.

Ammonium Sulphate, 1.2 M: 15.858 g B.D.H. "Analar" $(NH_4)_2SO_4$ dissolved in about 90 ml of water, volume adjusted to 100 ml with water and solution filtered.

Calcium Chloride, 0.1 M: B.D.H. "Analar" $CaCl_2$ (Dried) dried at 150°C for about 18 hours, 11.099 g taken and dissolved in about 900 ml of water, volume adjusted to 1000 ml with water, solution divided into 50 ml aliquots in multidose containers, and sterilized in an autoclave (10 Lb./sq.in. for 30 minutes).

Copper Sulphate, 0.02 M: 4.9942 g B.D.H. "Analar" $CuSO_4 \cdot 5H_2O$ dissolved in water and volume adjusted to 1000 ml with water.

Fiske and Subarrow Mixture (FSM): (Both solutions stored at 4°C).

(a) Ammonium Molybdate Solution: 2.5 g B.D.H. "Analar" $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ dissolved in water and volume made up to 100 ml with water.

(b) Reducing Reagent: 2.4 g B.D.H. "Analar" $Na_2SO_3 \cdot 7H_2O$ and 10.9696 g B.D.H. "Reagent Grade"

$Na_2S_2O_5$ dissolved in about 90 ml of water. 0.200 g B.D.H. "Reagent Grade" $NH_2C_{10}H_5(OH)SO_3H$ added to this solution and the mixture put on a mechanical shaker for 30 minutes. Volume then adjusted to 100 ml with water and solution filtered.

To make FSM, 2 volumes of (a) and 1 volume of (b) were combined on the day of use and kept at 0°C.

Magnesium Chloride, 1 M: 203.33 g B.D.H. "Analar" $MgCl_2 \cdot 6H_2O$ dissolved in water, volume adjusted to 1000 ml with water, solution divided into 50 ml aliquots in multidose containers, and sterilized in an autoclave as for Calcium Chloride.

Potassium Cyanide, 0.01 M: 0.6511 g B.D.H. "Analar" KCN dissolved in water and made up to 1000 ml with water.

Perchloric Acid, 0.05 M: 4.1 ml B.D.H. "Analar" 71-73 % W/W $HClO_4$ made up to 1000 ml with water.

Perchloric Acid, 0.15 M: 12.3 ml B.D.H. "Analar" 71-73 % W/W $HClO_3$ made up to 1000 ml with water.

Perchloric Acid, 0.3 M: 24.6 ml B.D.H. "Analar" 71-73 % W/W $HClO_3$ made up to 1000 ml with water.

Sodium Chloride, 0.9 %: Evans Medical Ltd., Sodium Chloride Injection B.P. used.

Sodium Hydroxide, 1 N: B.D.H. Volumetric Solution of NaOH, 1 N, used.

Sodium Pentobarbitone: Nembutal (60 mg/ml) used.

Standard Inorganic Phosphate: 16.3308 g B.D.H. "Analar" KH_2PO_4 dissolved in water and volume adjusted to 100 ml with water. This solution then diluted 1 in 100 in 0.05 M PCA to give a 12 µmole/ml solution. Further dilutions of 1 ml, or less, in 100 ml 0.05 M PCA were made and 10 ml used for the assay of inorganic phosphate as described in the "Methods" section of this thesis.

Standard Phosphocreatine, 5 µmoles/ml: 0.0327 g Sigma Phosphocreatine $\cdot 2Na \cdot 4H_2O$ dissolved in ice-cold water, volume adjusted to 20 ml, and 1 ml aliquots frozen and stored at -15°C. One ml diluted to 100 ml with 0.05 M PCA and 10 ml used for the assay

of phosphocreatine as described in the "Methods" section of this thesis.

Succinate Buffer, 0.33 M, pH 6.1 (Kalckar, 1947b):

78.6 g B.D.H. "Analar" succinic acid dissolved in about 1000 ml of water, 122 ml 10 N NaOH added, volume adjusted to about 1950 ml with water, pH adjusted to 6.1 by adding 1 N NaOH, and volume then adjusted to 2000 ml with water. Solution divided into 100 ml aliquots in multidose containers and sterilized in an autoclave as for Calcium Chloride. Just before use, 1 volume of this solution combined with an equal volume of water.

Sulphuric Acid, 5 N: B.D.H. Volumetric Solution of H_2SO_4, 5 N, used.

APPENDIX II

STATISTICAL DESIGNS AND ANALYSIS

SIMPLE RANDOMIZATION OF TWO TREATMENTS (series 1)

The rats were selected in pairs of equal body weight and whether or not the drug or control animal of each pair was injected and the tissue removed and assayed first or second was determined using a table of random numbers. Equal chances were given for the drug or control to come first or second by arbitrarily deciding that a number between 0 and 24 in the table indicated the control was to come first while a number between 25 and 49 indicated that the drug was to come first. Thus the following design was obtained:

Group	1	Drug First; Control Second
	2	Drug First; Control Second
	3	Control First; Drug Second
	4	Drug First; Control Second
	5	Control First; Drug Second
	6	Drug First; Control Second
	7	Control First; Drug Second
	8	Control First; Drug Second
	9	Drug First; Control Second
	10	Control First; Drug Second

Calculation of Significance Using Analysis of Variance

As a sample the values for the ratio ATP/ADP are given, but the calculations for AMP, ADP, ATP and the total (AMP + ADP + ATP) are done in a similar way.

Group	Control	Drug Treated	Control + Drug Treated
1	1.40	2.51	3.91
2	0.92	1.66	2.58
3	1.42	3.60	5.02
4	2.05	2.38	4.43
5	1.83	3.07	4.90
6	1.66	4.13	5.79
7	1.72	4.71	6.43
8	1.37	2.87	4.24
9	2.27	4.55	6.82
10	3.26	3.58	6.84
Totals	17.90	33.06	50.96

(1) Total Sum of Squares

$= (1.40)^2 + (0.92)^2 + \text{----} + (3.26)^2 + (2.51)^2 \text{----} + (3.58)^2 - \text{C.F.}$, where C.F. = Correction Factor

$= \dfrac{(\text{Grand Total})^2}{\text{Total number of observations}}$

Therefore, total sum of squares $= 153.8474 - \dfrac{(50.96)^2}{20}$

$= 153.8474 - 129.8461 = \underline{24.0013}$

(2) Drug Versus Control Sum of Squares

$= \dfrac{(17.90)^2}{10} + \dfrac{(33.06)^2}{10} - \text{C.F.}$

$= 141.3374 - 129.8461 = \underline{11.4913}$

(3) Between Groups Sum of Squares

$= \dfrac{(3.91)^2}{2} + \dfrac{(2.58)^2}{2} + \text{---}$ to 10 terms $- \text{C.F.}$

$= 138.4624 - 129.8461 = \underline{8.6163}$

(4) Error Sum of Squares

$= (1) - (2) - (3) = 24.0013 - 11.4913 - 8.6163 = \underline{3.8937}$

For convenience, these calculated values are put into a table of the following form in order to determine if there are any significant differences.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F
Groups	9	8.6163	0.9574 (a)	(a)/(c) = 2.21
Drug Versus Control	1	11.4913	11.4913 (b)	(b)/(c) = 26.56
Error	9	3.8937	0.4326 (c)	
Total	19	24.0013		

From tables of the distribution of F:

1. Between groups with 9 degrees of freedom for the greater mean square (i.e. groups) and 9 degrees of freedom for the lesser mean square (i.e. error), F at 5 % level is 3.81. Since the F value found (2.21) is less than this, there is no significant difference between groups (e.g. F is less than $F_{0.05}$).

2. Between drug and control with 1 and 9 degrees of freedom respectively for the greater and lesser mean squares, F at 0.1 % level is 22.86. Since the F value found (26.56) is greater than this, the difference between the drug and control values is significant at the 0.1 % level (e.g. F is greater than $F_{0.001}$).

SIMPLE RANDOMIZATION OF THREE TREATMENTS
(Series 2)

In series 2, the rats were selected in groups of 3 of equal body weight. The order of treatment of the animals in each group with the control solution, d-amphetamine or l-amphetamine solutions was determined using a table of random numbers and assigning equal chances for each treatment to come first, second or third. The following statistical design, where C = control, l = l-amphetamine and d = d-amphetamine, was thus obtained.

Group	Order		
	First	Second	Third
1	l	C	d
2	C	d	l
3	l	C	d
4	C	d	l
5	d	C	l
6	d	l	C
7	l	d	C
8	d	l	C
9	C	l	d
10	d	C	l

The analysis of variance was similar to that used for series 1 except the degrees of freedom were changed. As an example, the analysis of variance table for the ATP values is given.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F
Between Groups	9	0.3259	(a) 0.0362	a/c = 1.34
Control versus l-amphetamine versus d-amphetamine	2	0.5480	(b) 0.2740	(b)/(c) = 10.14
Error	18	0.4864	(c) 0.02704	
Total	29	1.3603		

From tables of distribution of F:

1. Between groups: $F_{0.05} = 3.55$. Since F less than $F_{0.05}$, there is no significant difference between groups.

2. Between Control and Drug treatments: $F_{0.01} = 6.01$. Since F is greater than $F_{0.01}$, there is a difference between the treatments at the 1 % level.

If a difference between two means is significant it must be greater than or equal to:

$$2.101 \sqrt{0.02702\ (1/10 + 1/10)} = 0.1544$$

	Control	d-amphetamine	l-amphetamine
Mean Value	2.115	2.395	2.102

Thus the significant difference is between the control and d-amphetamine values or between the l-amphetamine and d-amphetamine values. There is no significance between the control and l-amphetamine values.

The standard error of a single observation

$$= \sqrt{0.02702} = 0.1644$$

$$\text{Grand mean} = \frac{66.12}{30} = 2.2040$$

$$\text{Coefficient of Variation} = \frac{0.1644}{2.2040} \times 100 = 7.46\ \%$$

3 x 3 LATIN SQUARE DESIGN

(series 3 to 8 and 11 to 25 inclusive)

In each of these series, the rats were selected in groups of 3 of equal body weight. One rat in each group acted as a control while the other two were treated with drugs. The order of treatment and all subsequent procedures was randomized by using three of the five possible 3 x 3 latin squares. As an example, the statistical design for series 4 and the analysis of variance of the ATP values are shown. In this series, the effects of 0.90 mg/kg l-ephedrine (E) and 0.90 mg/kg dl-phenmetrazine (P) were investigated.

Statistical Design

	Column 1	Column 2	Column 3
Group 1 (row 1)	C	P	E
Group 2 (row 2)	E	C	P
Group 3 (row 3)	P	E	C
	Column 4	**Column 5**	**Column 6**
Group 4 (row 4)	P	E	C
Group 5 (row 5)	E	C	P
Group 6 (row 6)	C	P	E
	Column 7	**Column 8**	**Column 9**
Group 7 (row 7)	E	C	D
Group 8 (row 8)	P	E	C
Group 9 (row 9)	C	P	E

The data thus obtained is arranged in a table according to the treatment and in tables in the same order as it was obtained.

Group or Row	Control	E	P
1	2.20	2.15	2.47
2	2.15	2.14	2.19
3	2.17	1.96	2.20
4	2.15	1.96	2.42
5	2.21	2.46	1.94
6	1.77	2.03	2.36
7	1.71	1.72	2.13
8	1.69	1.51	2.31
9	2.32	2.01	2.18
Total	18.37	17.94	20.20
Mean	2.04	1.99	2.24

	Column 1	Column 2	Column 3	Totals
row 1	2.20	2.47	2.15	6.82
row 2	2.14	2.15	2.19	6.48
row 3	2.20	1.96	2.17	6.33
Totals	6.54	6.58	6.51	19.63

	Column 4	Column 5	Column 6	Totals
row 4	2.42	1.96	2.15	6.53
row 5	2.46	2.21	1.94	6.61
row 6	1.77	2.36	2.03	6.16
Totals	6.65	6.53	6.12	19.30

	Column 7	Column 8	Column 9	Totals
row 7	1.72	1.71	2.13	5.56
row 8	2.31	1.51	1.69	5.51
row 9	2.32	2.18	2.01	6.51
Totals	6.35	5.40	5.83	17.58

1. Total Sum of Squares

$= (2.20)^2 + \text{----} (2.32)^2 + (2.15)^2 + \text{----} (2.01)^2 + (2.47)^2$

$+ \text{----} (2.18)^2 - \text{C.F.}$ where $\text{C.F.} = \frac{(\text{Grand Total})^2}{27}$

$= \frac{(56.51)^2}{27} = 118.2733$

Therefore Total sum of squares $= 119.8619 - 118.2733$

$= \underline{\underline{1.5886}}$

2. Treatment Sum of Squares (i.e. control versus E versus P)

$= \frac{(18.37)^2 + (17.94)^2 + (20.20)^2}{9} - \text{C.F.}$

$= 118.5934 - 118.2733 = \underline{\underline{0.3201}}$

3. Blocks Sum of Squares (i.e. Between Latin Squares)

$$= \frac{(19.63)^2 + (19.30)^2 + (17.58)^2}{9} - \text{C.F.}$$

$$= 118.5426 - 118.2733 = \underline{0.2693}$$

For rows and columns sum of squares the first term (118.5426) becomes the Correction factor.

4. Rows Sum of Squares

$$= \frac{(6.82)^2 + (6.48)^2 + (6.33)^2 + (6.53)^2 + (6.61)^2 + (6.16)^2}{3}$$

$$+ \frac{(5.56)^2 + (5.51)^2 + (6.51)^2}{3} - 118.5426 = \underline{0.2916}$$

5. Columns Sum of Squares

$$= \frac{(6.54)^2 + (6.58)^2 + (6.51)^2 + (6.65)^2 + (6.53)^2 + (6.12)^2}{3}$$

$$+ \frac{(6.53)^2 + (5.40)^2 + (5.83)^2}{3} - 118.5426 = \underline{0.2030}$$

6. Error Sum of Squares

$$= (1) - (2) - (3) - (4) - (5)$$

$$= 1.5886 - 0.3201 - 0.2693 - 0.2916 - 0.2030 = \underline{0.5043}$$

Analysis of Variance

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F
Blocks	2	0.2693	0.1346	2.12
Rows	6	0.2919	0.0487	< 1
Columns	6	0.2030	0.0338	< 1
Treatments	2	0.3201	0.1600	3.17
Error	10	0.5043	0.0504	
Total	26	1.5886		

Significance

1. Blocks: F is less than $F_{0.05}$.

   Therefore no significance.

2. Treatments: F is less than $F_{0.05}$.

   Therefore no significance.

The standard error of a single observation $= \sqrt{0.05043}$

$= 0.2246$

Grand mean $= \frac{56.51}{27} = 2.0930$

Coefficient of Variation $= \frac{0.2246}{2.0930} = 10.73\ \%$

STATISTICAL DESIGN - DOUBLE RANDOMIZATION
(series 9)

The rats were selected in pairs and whether the control or drug treatment came first or second was determined using a table of random numbers and assigning equal chances for each treatment to come first or second. The effects of the drug at 3 different times after injection ($\frac{1}{2}$, $1\frac{1}{2}$ & 3 hours) was studied. In order that a direct comparison could be made between the effects at these three times, the order of treatment of the pairs of rats at each time interval was randomized using the 3 x 3 latin square design. Thus the following statistical design, where C_1 = $\frac{1}{2}$ hr. control, C_2 = $1\frac{1}{2}$ hr. control, C_3 = 3 hr. control, D_1 = $\frac{1}{2}$ hr. drug, D_2 = $1\frac{1}{2}$ hr. drug, and D_3 = 3 hr. drug, was obtained:

C_1	D_1	D_3	C_3	C_2	D_2
C_2	D_2	C_1	D_1	C_3	D_3
C_2	D_3	D_2	C_2	C_1	D_1

D_3	C_3	C_2	D_2	D_1	C_1
C_1	D_1	D_3	C_3	D_2	C_2
D_2	C_2	D_1	C_1	C_3	D_3

C_1	D_1	D_2	C_2	C_3	D_3
C_2	D_2	C_3	D_3	D_1	C_1
D_3	C_3	C_1	D_1	C_2	D_2

It is apparent that all of the control values make up a 3 x 3 latin square design and accordingly they were analyzed in the manner previously described (pages xi - xiv) to find out if there was any significant difference between the values obtained at the three different time intervals. Similarly, all of the drug values were analyzed to find out if there was any significant difference between the values obtained at the three different times. In no case was any significant difference found between the three different times.

Since the pairs from the three different time intervals also form a 3 x 3 latin square design, the difference between the drug and control values at each time interval was similarly analyzed to find out if there was any significant difference between the drug and control values. As an example of this, the analysis of variance table for the control minus the drug values for ADP is shown below.

Variation due to	Degrees of Freedom	Sum of Squares	Mean Square	F
Blocks	2	0.0020	0.0010	1
Rows	6	0.0514	0.0086	1
Columns	6	0.1012	0.0169	1.44
½ hr. versus 1 hr. versus 3 hr.	2	0.0692	0.0345	2.95
Error	10	0.1174	0.0117	
Total	26	0.3412		

There are no significant F values.

From the table, the standard error of the difference between the control and drug values for ADP equals $\sqrt{0.01174}$. If a mean difference between the control and drug value is to be significantly different from zero (on the 5 % level of significance) it must be greater than or equal to

$$2.228 \sqrt{\frac{0.01174}{9}} = 0.081$$

(2.228 is the 't' value for error degrees of freedom of 10 at the 5 % level of significance. The 't' value at any other level of significance may be substituted to find the appropriate level of significance).

In the present case of ADP values the mean differences at the different times were:

$C_1 - D_1 = 0.144$ (i.e. $\frac{1}{2}$ hour)
$C_2 - D_2 = 0.065$ (i.e. $1\frac{1}{2}$ hour)
$C_3 - D_3 = 0.188$ (i.e. 3 hours)

Therefore, $\frac{1}{2}$ hour and 3 hours after treatment, the drug treated animals have a significantly lower ADP level than the control animals (at the 5 % level of significance).

Using the 't' value of 3.169 for the 1 % level of significance, a difference would have to be greater than 0.112 to be significant at this level.

Using the 't' value of 3.169 for the 1 % level of significance, a difference would have to be greater than 0.112 to be significant at this level.

Using the 't' value of 4.587 for the 0.1 % level of significance, a difference would have to be greater than 0.165 to be significant.

Therefore, the difference $C_1 - D_1$ was significant at the 1 % level (i.e. $0.01 > P > 0.001$). The difference $C_3 - D_3$ was significant at the 0.1 % level (i.e. $0.001 > P$). In other words, the ADP levels in the drug-treated rats were significantly lower than the control levels $\frac{1}{2}$ and 3 hours after treatment.

DOUBLE RANDOMIZATION - THREE TREATMENTS
(series 10)

The statistical design in this series was similar to that in series 9 (page xv) with the exception that there were three treatments at each of the three time intervals. The rats were, therefore, selected in groups of 3 of equal body weight. At each time interval the order of treatment of the 3 rats with the control solution (C), d-amphetamine solution (D_1) or dl-phenmetrazine solution (D_2) was randomized using three 3 x 3 latin squares. In order that a direct comparison could be made between the values obtained after each of these treatments at the three different time intervals (T_1, T_2 & T_3), the order in which the groups of three was done was randomized using three 3 x 3 latin squares. Thus the following statistical design was obtained:

T_2 (C D_1 D_2)	T_1 (C D_1 D_2)	T_3 (D_2 C D_1)
T_3 (D_1 D_2 C)	T_2 (D_2 C D_1)	T_1 (D_1 D_2 C)
T_1 (D_2 C D_1)	T_3 (C D_1 D_2)	T_2 (D_1 D_2 C)

T_1 (D_2 C D_1)	T_2 (D_1 C D_2)	T_3 (D_1 D_2 C)
T_3 (C D_1 D_2)	T_1 (D_1 D_2 C)	T_2 (D_2 D_1 C)
T_2 (C D_2 D_1)	T_3 (D_2 C D_1)	T_1 (C D_1 D_2)

T_1 (D_1 D_2 C)	T_3 (C D_1 D_2)	T_2 (D_2 D_1 C)
T_3 (D_2 C D_1)	T_2 (C D_2 D_1)	T_1 (C D_1 D_2)
T_2 (D_1 C D_2)	T_1 (D_2 C D_1)	T_3 (D_1 D_2 C)

Each set of values obtained (i.e., inorganic phosphate, phosphocreatine, AMP, ADP, ATP, AMP + ADP + ATP and ATP/ADP ratio) was analyzed in the following six ways for significance.

1. All of the T_1 values form three 3 x 3 latin squares and were analyzed in the manner previously described for such a design to find out if there was any difference between C_1, D_1 or D_2 at this time interval.
2. All of the T_2 values were similarly analyzed to find out if there was any difference between C, D_1 or D_2 at this time interval.
3. All of the T_3 values were similarly analyzed to find out if there was any difference between C, D_1 or D_2 at this time interval.
4. Since T_1, T_2 and T_3 were also randomized using three 3 x 3 latin squares, all of the control values may be taken and analyzed in the normal way to find out if there was any difference between the control values at the three different time intervals.
5. Similarly, all of the D_1 values were analyzed to find out if there was any difference between the values at the three different time intervals.
6. Similarly, all of the D_2 values were analyzed to find out if there was any difference between the values at the three different time intervals.

EXPERIMENTAL PROTOCOL

(Series 7)

C = 0.9 % Saline; P = dl-Phenmetrazine(0.90 mg/ml); D = d-Phendimetrazine(0.90 mg/ml)

Date (1961)	Group	Weight (g)	Treatment	Time of Treatment	Time of Killing	Time of Dissection	Date (1961)	Time of Extraction	Time Assay Begun
24:7		75	0.15 ml C	8:10 a.m.	12:10 p.m.	12:30 p.m.	24:7	1:45 p.m.	2:15 p.m.
	1	75	0.15 ml P	8:11 a.m.	12:11 p.m.	12:45 p.m.		2:30 p.m.	3:05 p.m.
		75	0.15 ml D	8:12 a.m.	12:12 p.m.	1:00 p.m.		3:20 p.m.	3:55 p.m.
24:7		75	0.15 ml P	1:34 p.m.	5:34 p.m.	6:05 p.m.	25:7	8:30 a.m.	9:05 a.m.
	2	75	0.15 ml D	1:35 p.m.	5:35 p.m.	6:15 p.m.		9:25 a.m.	9:55 a.m.
		75	0.15 ml C	1:36 p.m.	5:36 p.m.	6:25 p.m.		10:30 a.m.	11:00 a.m.
24:7		75	0.15 ml D	1:40 p.m.	5:40 p.m.	6:45 p.m.	25:7	1:00 p.m.	1:35 p.m.
	3	75	0.15 ml C	1:41 p.m.	5:41 p.m.	7:00 p.m.		1:55 p.m.	2:25 p.m.
		75	0.15 ml P	1:42 p.m.	5:42 p.m.	7:10 p.m.		2:45 p.m.	3:20 p.m.
25:7		75	0.15 ml D	12:45 p.m.	4:45 p.m.	5:25 p.m.	26:7	8:15 a.m.	8:55 a.m.
	4	75	0.15 ml P	12:46 p.m.	4:46 p.m.	5:35 p.m.		9:10 a.m.	9:45 a.m.
		75	0.15 ml C	12:47 p.m.	4:47 p.m.	5:50 p.m.		10:05 a.m.	10:40 a.m.
25:7		75	0.15 ml P	12:50 p.m.	4:50 p.m.	6:00 p.m.	26:7	1:30 p.m.	2:05 p.m.
	5	75	0.15 ml C	12:51 p.m.	4:51 p.m.	6:15 p.m.		2:20 p.m.	2:50 p.m.
		75	0.15 ml D	12:52 p.m.	4:52 p.m.	6:25 p.m.		3:15 p.m.	3:45 p.m.
26:7		75	0.15 ml C	1:10 p.m.	5:10 p.m.	6:00 p.m.	27:7	8:30 a.m.	9:10 a.m.
	6	75	0.15 ml D	1:11 p.m.	5:11 p.m.	6:10 p.m.		9:30 a.m.	10:00 a.m.
		75	0.15 ml P	1:12 p.m.	5:12 p.m.	6:20 p.m.		10:20 a.m.	10:55 a.m.
26:7		80	0.16 ml P	1:17 p.m.	5:17 p.m.	6:40 p.m.	27:7	1:25 p.m.	2:00 p.m.
	7	80	0.16 ml D	1:18 p.m.	5:18 p.m.	6:55 p.m.		2:15 p.m.	2:50 p.m.
		80	0.16 ml C	1:19 p.m.	5:19 p.m.	7:05 p.m.		3:15 p.m.	3:50 p.m.
27:7		85	0.17 ml D	1:07 p.m.	5:07 p.m.	5:45 p.m.	28:7	8:30 a.m.	9:10 a.m.
	8	85	0.17 ml C	1:08 p.m.	5:08 p.m.	6:00 p.m.		9:25 a.m.	10:00 a.m.
		85	0.17 ml P	1:09 p.m.	5:09 p.m.	6:20 p.m.		10:20 a.m.	10:55 a.m.
27:7		75	0.15 ml C	1:14 p.m	5:14 p.m.	6:40 p.m.	28:7	1:30 p.m.	2:05 p.m.
	9	75	0.15 ml P	1:15 p.m.	5:15 p.m.	6:50 p.m.		2:25 p.m.	3:00 p.m.
		75	0.15 ml D	1:16 p.m.	5:16 p.m.	7:00 p.m.		3:45 p.m.	4:20 p.m.