STUDIES ON THE MODE OF ACTION OF CENTRALLY-ACTING DRUGS

A thesis submitted to the University of Glasgow in candidature for the degree of

Doctor of Philosophy

in the

Faculty of Science

by

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I would like to express my sincere appreciation to the many people who have helped me during my studies.

I wish to thank Professor R.C. Garry, Regius Professor of Physiology, for the opportunity to carry out this research and also for his guidance and valuable advice throughout my investigations.

I would like to thank Professor S. Alstead, C.B.E., Regius Professor of Materia Medica for permitting me to use the facilities of his department.

I would like to record my sincere gratitude to the late Mr. J.J. Lewis, Senior Lecturer in Experimental Pharmacology. I will always remember and appreciate his kindly interest and encouragement.

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David Pollock.

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Publications

Certain aspects of the work described in this thesis have been either published jointly with the late Mr. J.J. Lewis or have been presented as communications at scientific meetings.

Lewis, J.J. & Pollock, D. (1965). Effects of d-amphetamine and chlorpromazine on oxidised (NAD) and reduced ($NADH_2$) nicotinamide adenine dinucleotide levels in rat brain. Biochem. Pharmac. 14, 636-638.

Lewis, J.J. & Pollock, D. (1965). Effects of some centrally-acting drugs on brain levels of NAD and $NADH_2$ in the rat. A paper presented to the British Pharmacological Society - Winter Meeting, London, 5th to 7th January, 1965.

Pollock, D. (1965). Influence of drugs on energy-linked reactions in the brain. A paper presented to a Symposium on Psychopharmacology - Edinburgh, 8th to 9th October, 1965.

In addition, the following paper, on a related topic, was published.

Effects of drugs on rat brain *in vitro* respiration and adenosinetriphosphatase activity. *Life Sciences* **4**, 21-26.

Conventions for Citing References

The conventions used in this thesis for citing references are those of the Journal of Physiology. When the abbreviation for a journal did not appear in the "Suggestions to Authors" (J. Physiol. (1966), 182, 1-33) or in the "World Medical Periodicals" (published by the World Medical Association, New York, 1957) the full title of the journal was used.

General Introduction

General Introduction

Philosophical speculation has long been focussed upon the relationship between mind and brain (Roth, 1958; Walshe, 1959; Brain, 1963; Clarke, 1963). However, despite the traditional use of drugs for modifying behaviour and experience (Goodman & Gilman, 1956; Eiduson, Geller, Yuwiler & Eiduson, 1964a), little was known until recently of either the basic nature of brain functioning or of the mechanisms of drug action upon it. In the past, this ignorance was partly a reflection of man's reluctance to study himself with the same enthusiasm and detachment, that he has applied so successfully to the investigation of his external environment (Walker, 1961). Its persistence today is mainly due to the extreme complexity of the brain. Interest has now turned from this age-old preoccupation with the enigma of the mind, towards the more practical, mechanistic view (Cohen, 1952), that brain function is the result of a multitude of simultaneous, related, chemical and physical events (Eiduson et al, 1964b).

Such a concept provides a valuable framework

for interpreting the pharmacodynamic activities of psychotropic drugs. Although the experimental evidence is incomplete, and often conflicting, certain aspects of brain function and drug action have been associated with particular physico-chemical phenomena (Richter & Crossland, 1949; Crossland & Merrick, 1954). Nevertheless, extensive research is still required to elucidate the precise mechanisms of action of these drugs. This is emphasised by several factors. There is confusion surrounding the classification of psychotropic drugs (Jacobsen, 1959; 1963; Shepherd & Wing, 1962) and even scepticism concerning their therapeutic efficacy (Jarvik, 1958; Inglis, 1965). Furthermore, it is undesirable that chance should still be an important element in their discovery (Robson & Stacey, 1962).

In attempts to understand the actions of these drugs, data from various experimental approaches have been examined and evaluated. Information obtained exclusively from a single technique is of limited value, unless it can be correlated with, or corroborated by

observations from another field. For example, behavioural studies in humans and animals provide only suggestive evidence concerning the primary sites or mechanisms of drug action.

Electrophysiological techniques have been used with limited success. For instance, amphetamine initiates in the electroencephalogram (EEG) of experimental animals, fast low voltage waves, which correspond to the period of behavioural arousal (Schallek & Walz, 1953; Rinaldi & Himwich, 1955; Longo & Silvestrini, 1957a,b; Monnier, 1957; Himwich, 1959; Killam, 1962). This response is absent when the integrity of the functionally-related reticular activating system (Birzis, 1960; French, 1960) is abolished (Elkes, Elkes & Bradley, 1954; Konigsmark, Killam & Killam, 1958; Killam, Gangloff, Konigsmark & Killam, 1959). Interpretation of these observations is however difficult, since amphetamine-induced EEG desynchrony can be inhibited, while behavioural arousal persists (Bradley, 1958). Neurophysiological studies with other psychotropic drugs have been even less conclusive, since it is difficult to

associate behavioural effects with particular patterns of electrical activity in specific areas of the brain (Bradley & Key, 1958; Konigsmark et al, 1958; Costa, Pscheidt, Van Meter & Himwich, 1960; Funderburk, Finger, Drakontides & Schneider, 1962).

Many attempts have been made to establish a relationship between the pharmacological activities of centrally-acting drugs and modification of the metabolism of hypothetical transmitters within the brain (Brodie, Olin, Kuntzman & Shore, 1957; Zeleny, Lindaur & Kozak, 1957; Gey & Pletscher, 1961; De Ropp & Snedeker, 1961; Ganrot, Rosengren & Gottfries, 1962; Giarman & Pepeu, 1962; McGeer, McGeer & Wada, 1963; Giarman & Pepeu, 1964). The validity of these studies depends largely upon whether or not the substance, assumed to have a transmitter function, satisfies the necessary criteria for such a role (Paton, 1958; Crossland, 1960; Toman, 1963). Supporting evidence for acetylcholine is particularly strong (Stedman & Stedman, 1937; MacIntosh, 1941; Feldberg & Vogt, 1948; Burgen & Chipman, 1951; Koelle, 1954; Hebb & Whittaker, 1958;

Whittaker, 1959; Aldridge & Johnson, 1959; De Robertis, Rodriguez de Lores Arnaiz, Salganicoff, Pellegrino de Iraldi & Zieher, 1963). Yet, most of the interest has been directed towards the catecholamines and 5-hydroxytryptamine, for which there is less satisfactory evidence (Von Euler, 1946; Holtz, 1950; Twarog & Page, 1953; Vogt, 1954; Amin, Crawford & Gaddum, 1954; Gaddum & Giarman, 1956; Montagu, 1956; Carlsson, Lindqvist, Magnusson & Waldeck, 1958; Rosengren, 1960; Potter & Axelrod, 1962). Nevertheless, in order to rationalise the central effects of reserpine and the monoamine oxidase blocking antidepressives, noradrenaline and 5-hydroxytryptamine have been postulated as neurohormones in the mutually-antagonistic ergotropic and trophotropic systems of Hess (1954) (Brodie & Shore, 1957; Brodie, 1958). Apart from a few isolated observations (Elliott & Van Gelder, 1960), less interest has been shown in the effects of drugs on other hypothetical transmitters (Kwiatkowski, 1943; Hellauer & Umrath, 1948; Florey, 1954; Crossland & Mitchell, 1956; Berl & Waelsch, 1958; Holton, 1959; Crossland, 1960).

Until the precise functions of these substances within the brain have been established, attempts to explain the mechanisms of psychotropic drug action on this basis will be unsatisfactory.

The fundamental importance of energy metabolism in brain function has resulted in numerous attempts to associate drug action with alterations in the basic pathways of energy production and utilisation (Butler, 1950; Quastel, 1952; McIlwain, 1959; 1962). Several correlations exist between various biochemical, behavioural and mental parameters (Woolley, 1962). In particular, the role of the nicotinamide nucleotides is especially interesting, since they represent the first link in the electron-transfer chain and the metabolism of their precursor, nicotinic acid, is implicated in a number of abnormal mental states (Agnew & Hoffer, 1955; Hoffer, Osmond, Callbeck & Kahan, 1957). The availability of evidence from several diverse sources, together with the established importance of these nucleotides in energy metabolism, suggest that the relationship between this aspect of brain function and psychotropic drug action is worthy of further investigation.

Specific Introduction

Part I

The Distribution and Metabolism of the Nicotinamide Coenzymes.

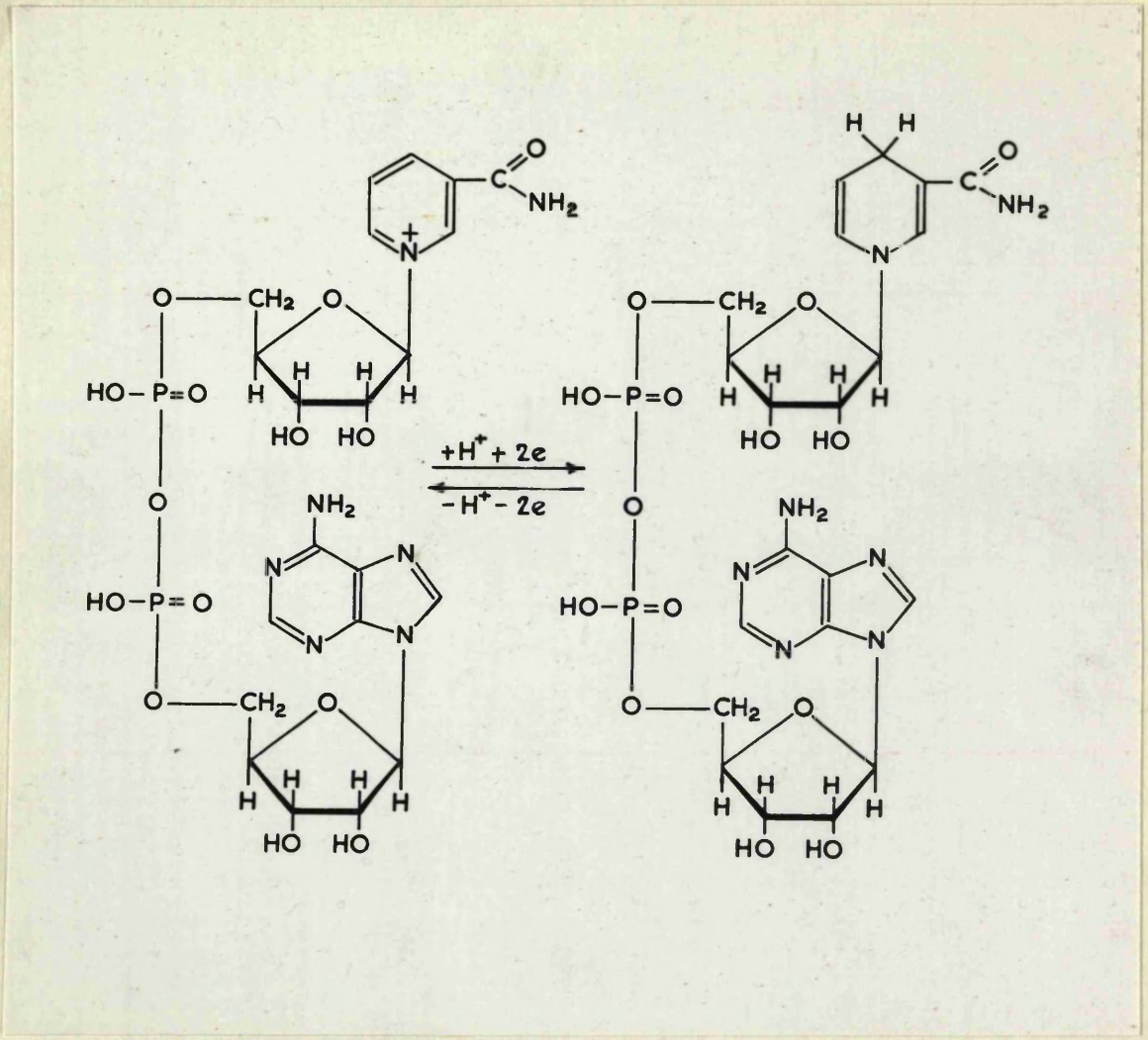

Fig. 1. Cyclic Oxidation-Reduction Changes Within the NAD Molecule.

Within the brain the propagation of the nervous impulse depends upon the close functional association of several biological transducing, or energy transferring systems (Green & Fleischer, 1962). For example, neural transmission depends upon collaboration between the mitochondrion, which provides energy in an appropriate form, and the nerve cell membrane, which controls the relative ionic balance within the cell (Siekevitz & Potter, 1955; Goodwin, 1960). Energy transfer is basically a molecular phenomenon, dependent upon cyclic alterations in specialised compounds. The nicotinamide nucleotides represent important examples of this type of compound, since they are reversibly oxidised and reduced (Fig. 1, Fig. 2) as the first stage of the electron-transfer chain (Conn, 1960; Asimov, 1962).

Since the initial isolation and identification of the nicotinamide coenzymes (Warburg & Christian, 1931; 1936; Von Euler, Albers & Schlenk, 1936), their participation has been demonstrated in numerous enzymic reactions (Kaplan, 1960a). There are two nicotinamide coenzymes, which mediate the dehydrogenation

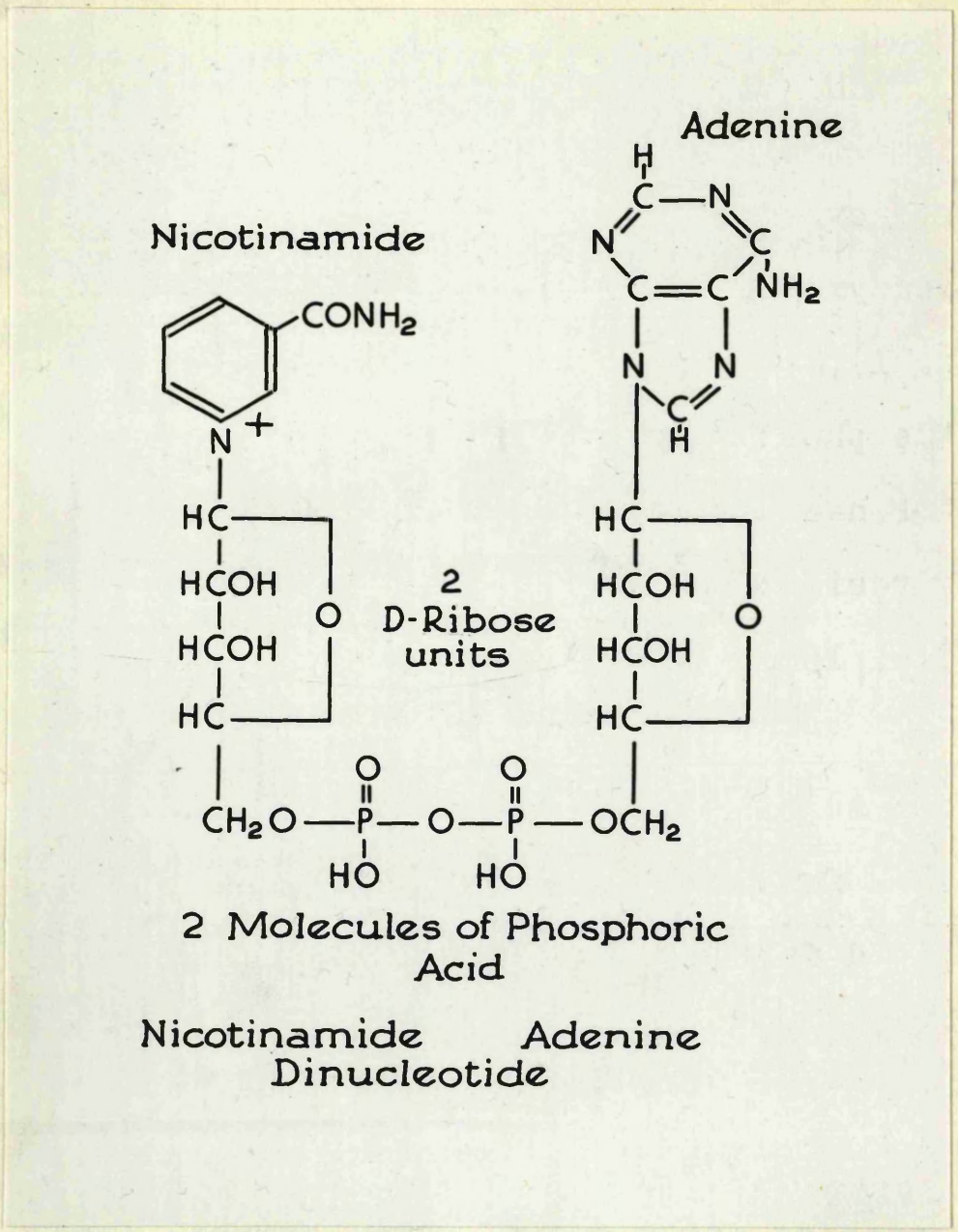

Fig. 3. Structural Formula of NAD, Showing the Main Components of the Molecule.

of most respiratory substrates. They are also involved in several biosynthetic transhydrogenations.

Coenzyme I, nicotinamide adenine dinucleotide (NAD) (Fig. 1), and coenzyme II, nicotinamide adenine dinucleotide phosphate (NADP) (Fig. 2), differ only in that NADP has a third phosphate monoester group on the ribose residue of adenylic acid (Kornberg & Pricer, 1950; Kaplan, 1960b; Kleiner & Orten, 1962; Needham, 1965) (Fig. 3).

NAD and NADP are valuable redox agents, since their reduced forms are excellent electron donors and their oxidised forms are almost equally good electron acceptors (Fig. 1, Fig. 2). These compounds owe their characteristic ability to be rapidly oxidised and reduced to specific steric arrangements within the nicotinamide moiety. This is reflected in the physical constants of these coenzymes (Szent-Gyorgyi, 1960; McElroy & Glass, 1961).

Although the exact mechanism of hydrogen transfer from substrate to coenzyme has not been determined, it is known to occur in a particular manner.

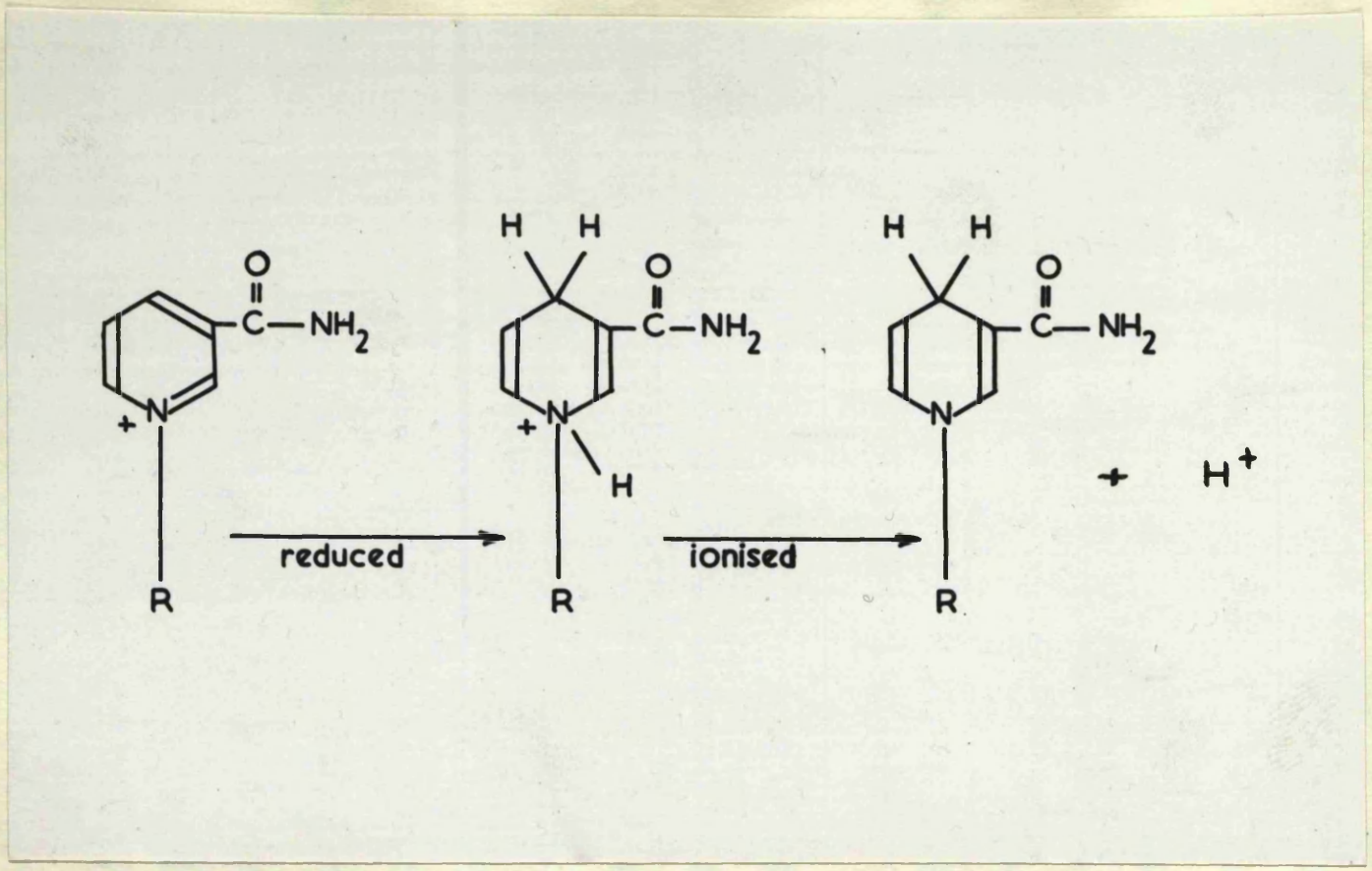

Fig. 4. Sequence of Changes Within the Nicotinamide Moiety of NAD and NADP During Reduction.

The specificity of this reaction, which has been confirmed by many investigations (Talalay, Dobson & Tapley, 1952; Pullman, San Pietro & Colowick, 1954; Burton & Kaplan, 1954; Vennesland & Westheimer, 1954; Hayes & Velick, 1954; Vennesland, 1956; Van Eys & Kaplan, 1957; Beckett, 1959; Conn, 1960; Wagner & Folkers, 1960) (Fig. 4), is associated with the spatial arrangement of atoms within the pyridine ring. Thus, during the oxidation of the general compound $R\text{-}CH_2OH$ one hydrogen atom is transferred from the terminal carbon to a position in the pyridine ring opposite the nitrogen. A second hydrogen atom, from the hydroxyl group, is transferred temporarily to the nitrogen, from which it is then rapidly released by ionisation (Fig. 4). A similar reaction also occurs during the nicotinamide coenzyme-catalysed oxidation of amino groups (Fig. 20). In addition, the planar structure of the nicotinamide moiety is important, since it introduces the possibility of individual enzymes reacting in a specific manner with different faces of this structure.

Despite the unique role of nicotinamide in the functioning of these coenzymes, this group may be replaced by other pyridine derivatives, with only slight modification in activity. Indeed, the use of NAD analogues has been valuable in understanding the special properties of these coenzyme molecules and in determining the basic mechanisms of action of various dehydrogenases (Weber & Kaplan, 1957; Lamborg, Stolzenbach & Kaplan, 1958; Van Eys, 1958; Van Eys, Stolzenbach, Sherwood & Kaplan, 1958; Anderson, Ciotti & Kaplan, 1959; Anderson & Kaplan, 1959; Shifrin & Kaplan, 1959; Strittmatter, 1959; Klingenberg & Bücher, 1960).

The nicotinamide coenzymes are widely distributed in nature (Kaplan, 1960a). However, the relative concentrations of NAD and NADP, and also the ratios of oxidised to reduced coenzymes, may vary considerably in different tissues (Glock & McLean, 1955; Jedeikin & Weinhouse, 1955; Jacobson & Kaplan, 1957; Jacobson & Astrachan, 1957). These differences in nicotinamide coenzyme content in various tissues, and indeed within the same organ at different times, may indicate adaptation

to physiological requirements (McLean, 1960). They may also reflect to some extent the inherent difficulties associated with measuring tissue nicotinamide coenzyme content (Klingenberg et al, 1960). Although large discrepancies exist between the various results obtained for tissue nicotinamide coenzyme levels, generally, it is found that organs engaged mainly in synthetic processes contain higher concentrations of NADP than NAD, while the reverse is true of tissues concerned primarily with oxidative energy production (Spirtes & Eichel, 1954; Glock et al, 1955; Lowry, Roberts & Kapphahn, 1957; Bassham, Birt, Hems & Loening, 1959).

Within the brain, oxidised NAD preponderates over all other forms of nicotinamide nucleotides (McIlwain, 1957). The total nicotinamide nucleotide content is however, much lower than that of other organs (Gore, Ibbott & McIlwain, 1950), but is less readily affected by either the administration, or inadequate dietary levels of the coenzyme precursor, nicotinamide (Brown, 1964; Bonasera, Mangione & Bonavita, 1965).

The relative concentrations of nicotinamide coenzymes also vary throughout the subcellular fractions

(Glock & McLean, 1956). For example, much higher concentrations exist in the mitochondria and cytoplasm than in the microsomes or the nucleus (Jacobson et al, 1957). The presence and firmness of binding of these coenzymes within the mitochondria suggest a functional differentiation at the subcellular level. This is further emphasised by the inaccessibility of intramitochondrial nicotinamide nucleotides to hydrolytic enzymes, present in other fractions (Slater, Bailie & Bouman, 1961). Such a separation into compartments within the cell, and possibly even within the mitochondria, may even be related to the functional integration of various energy-linked reactions. The relative rates of mitochondrial oxidative phosphorylation and glycolysis, within the cytoplasm, may thus depend upon the physical separation of the nicotinamide nucleotides into distinct subcellular "pools" (Lehninger, 1964).

The normal impermeability of the mitochondrial membrane towards specific substrates and essential coenzymes may be modified in certain circumstances. In in vitro experiments, a variety of substances,

including thyroxine, insulin and phosphate ions may provoke the loss of intramitochondrial pyridine coenzymes into the suspending medium. In this process, only the oxidised coenzymes are released, since the reduced forms remain firmly bound within the mitochondria.

Associated with these changes in coenzyme content, mitochondria also undergo changes in volume. These may be either passive, as a result of osmotic alterations, or active, and therefore dependent upon oxidative energy metabolism (Shaw, Lannon & Tapley, 1959; Lindberg, Löw, Conover & Ernster, 1961; Greenbaum & Dicker, 1963). The connection between mitochondrial swelling, nicotinamide nucleotide content and energy-linked reactions, is strengthened by the fact that both the morphological changes and the concomitant coenzyme leakage may be reversed by adenosinetriphosphate (ATP) (Lehninger, 1959; 1962; 1964).

These responses may be related to a number of *in vivo* observations. In the intact living cell, mitochondria continuously undergo changes in shape and

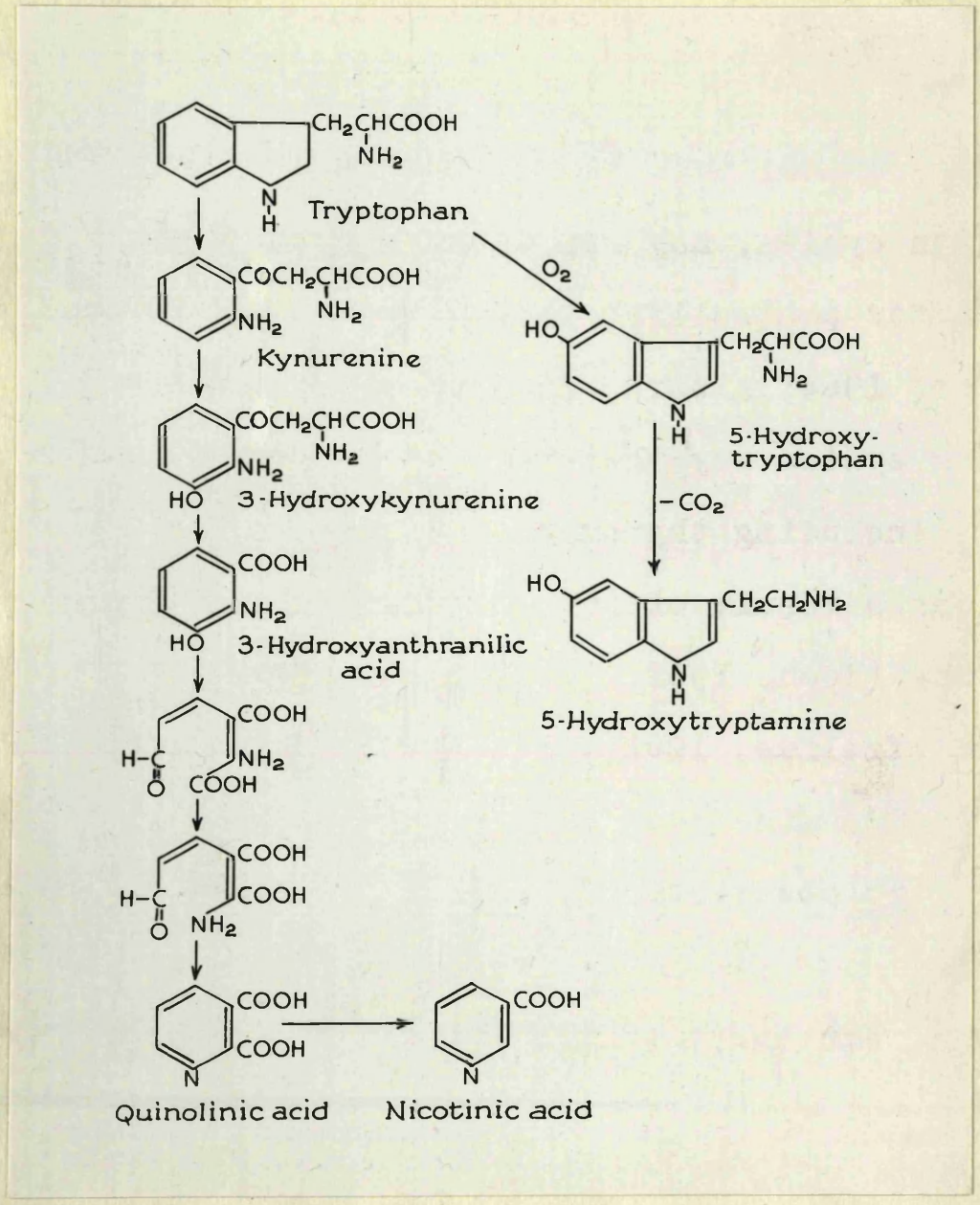

Fig. 5. Synthesis of Nicotinic Acid from Tryptophan, Showing the Related Biosynthetic Pathway of 5-Hydroxytryptamine.

volume. Such variations, consisting of swelling-contraction cycles, may represent a cybernetic mechanism for controlling the synthesis of ATP (Lehninger, 1964; Albert, 1965). This metabolic regulating system may therefore be modified by a variety of drugs, including thyroxine, and may be profoundly deranged as a result of inherent defects in mitochondrial functioning (Hoch, 1962; Luft, Ikkos, Palmieri, Ernster & Afzelius, 1962). Although the participation of the nicotinamide coenzymes may be inferred by analogy with *in vitro* observations, their precise role remains obscure.

The synthesis of the nicotinamide nucleotides depends upon the availability of adequate levels of the essential precursor, nicotinic acid, which may be derived either directly from the diet or indirectly, as a result of the conversion of dietary tryptophan, *via* the kynurenine pathway (Fig. 5) (Bonner & Yanofsky, 1951; Bessey, Lowe & Salomon, 1953; Birkinshaw, 1953; Musajo & Benassi, 1964). Thereafter, nicotinic acid is converted to nicotinamide, which combines with

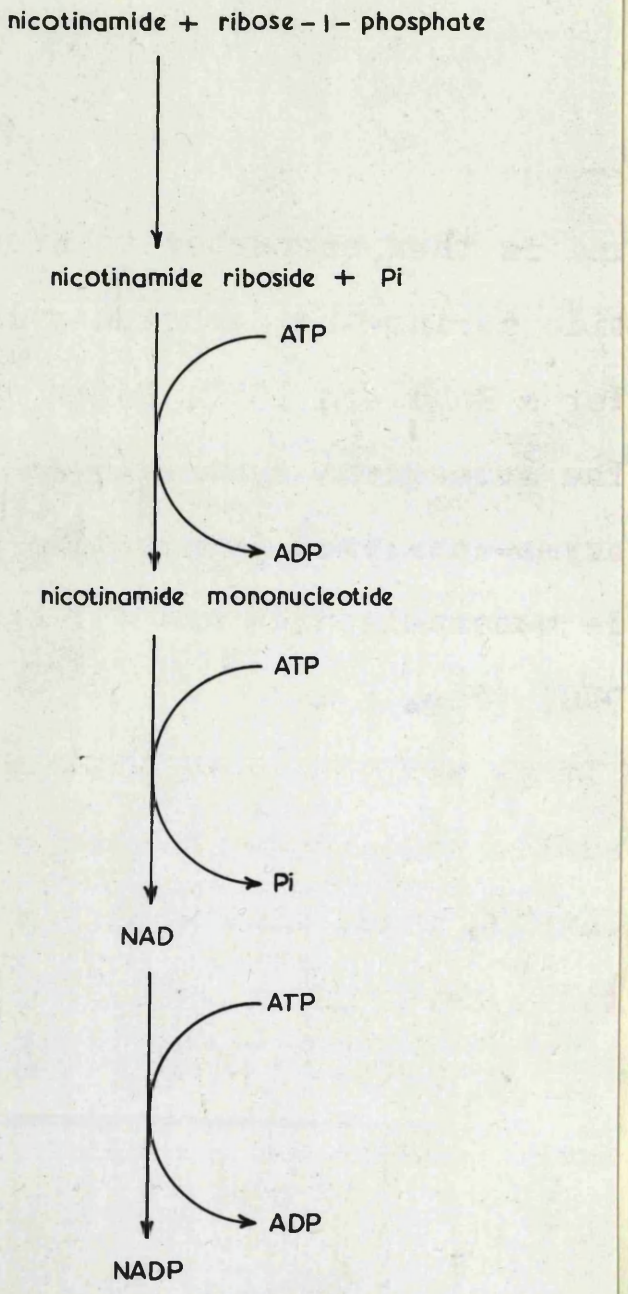

Fig. 6. Biosynthetic Pathway of the Nicotinamide Nucleotides.

ribose-1-phosphate to form nicotinamide riboside. This compound is then converted to nicotinamide mononucleotide during the dephosphorylation of ATP to ADP (Leder & Handler, 1951; Rowen & Kornberg, 1951) (Fig. 6). The subsequent synthesis of NAD depends upon the enzyme-catalysed interaction between nicotinamide mononucleotide and ATP (Korkes, del Campillo & Ochoa, 1950) (Fig. 6).

It is not yet known whether the synthesis of the nicotinamide nucleotides occurs within the brain (McIlwain, 1957), since no nicotinamide mononucleotides have been identified in brain tissue. In vitro studies indicate that isolated cerebral tissue does not synthesise NAD but can maintain its existing nicotinamide nucleotide content, if supplied with sufficient oxygen and glucose (Gore et al, 1950; McIlwain, Thomas & Bell, 1956).

The synthesis of NADP from NAD (Kornberg, 1950) (Fig. 6) and the enzymic reversal of this reaction also occur within the brain (Sanadi, 1952). The rate of

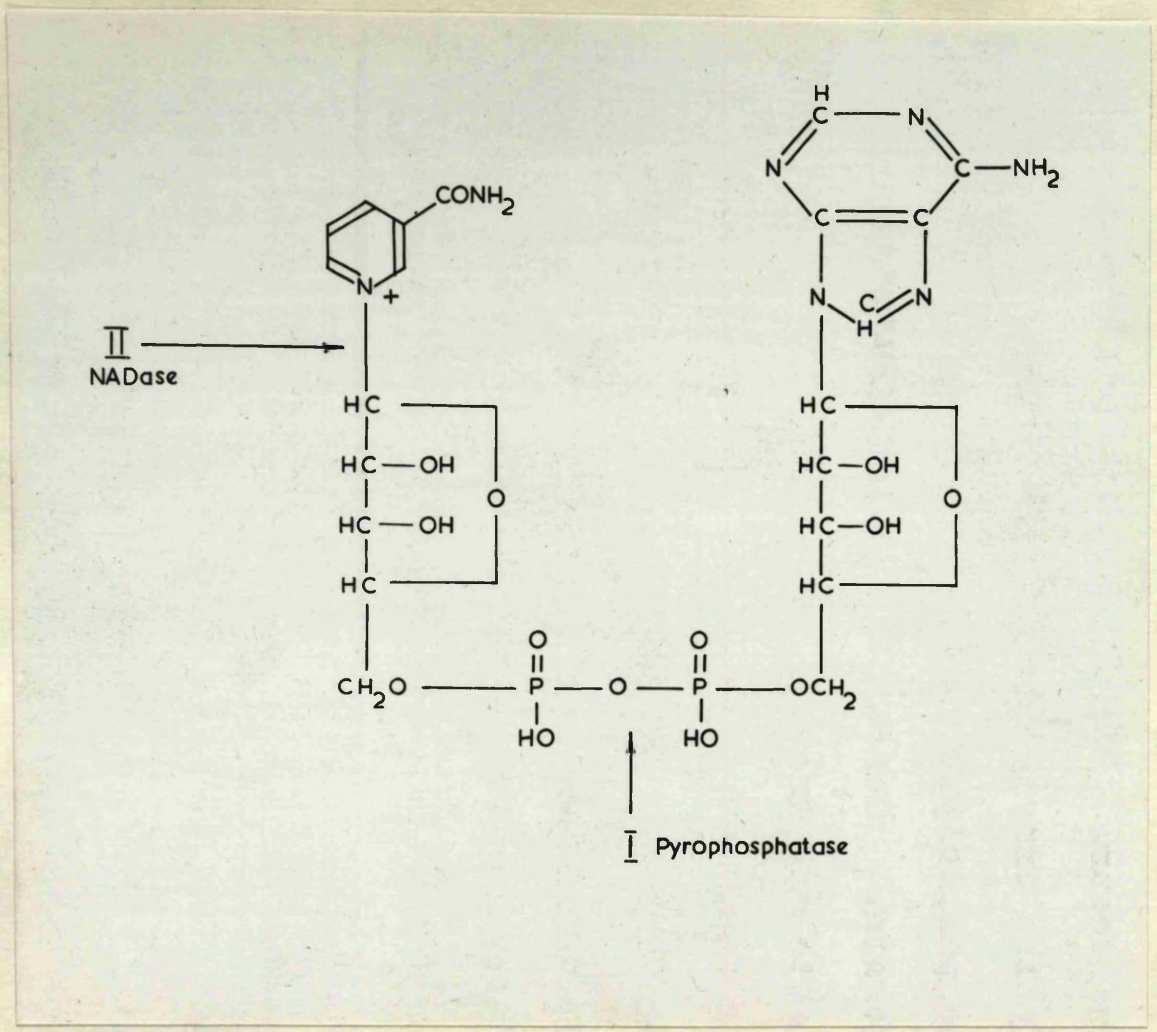

Fig. 7. Sites of Enzymic Hydrolysis Within the NAD Molecule.

interconversion of these nucleotides in cerebral tissue is however, much slower than in the liver.

A mutual oxidation-reduction between NAD and NADP may also occur. This reaction, which is catalysed by the enzyme, transhydrogenase, and requires the participation of ATP, is not prominent within the brain. Cerebral transhydrogenase can however promote the transfer of hydrogen between two molecules of NAD.

There are two enzymes involved in the hydrolysis of the nicotinamide nucleotides. One of these is responsible for rupturing the pyrophosphate bonds of NAD and NADP (Fig. 7). The second, which is generally referred to as NADase and is the only one found in nervous tissue (Jacobson & Kaplan, 1957), catalyses the cleavage of the nicotinamide riboside linkage (Fig. 7). Within the brain the distribution of NADase is uneven. High concentrations occur in the hypothalamus and thalamus, while smaller amounts are present in the cortex (Kaplan, 1960b). At the subcellular level NADase is mainly localised in the

microsomal fraction.

NADase participates not only in the cleavage of the nicotinamide moiety, but also in the exchange between free and coenzyme-bound nicotinamide. This latter mechanism may explain the inhibition of the hydrolytic component of brain NADase activity by nicotinamide (Mann & Quastel, 1941; Handler & Klein, 1942; McIlwain & Rodnight, 1949; McIlwain, 1950; Zatman, Kaplan & Colowick, 1953), which may alter the dynamic equilibrium, causing the exchange reaction to predominate (Kaplan, 1960b).

NADase also catalyses a non-specific exchange between various pyridine derivatives and coenzyme-bound nicotinamide (Anderson et al, 1959). It may even promote the incorporation of histamine into the NAD molecule (Alivisatos, 1958). Such a mechanism, involving the formation of NAD analogues, may explain the toxic effects of certain nicotinamide antagonists. This hypothesis is supported by various observations. For example, the marked neurological changes associated with 3-acetylpyridine toxicity (Fig. 23) occur mainly in those

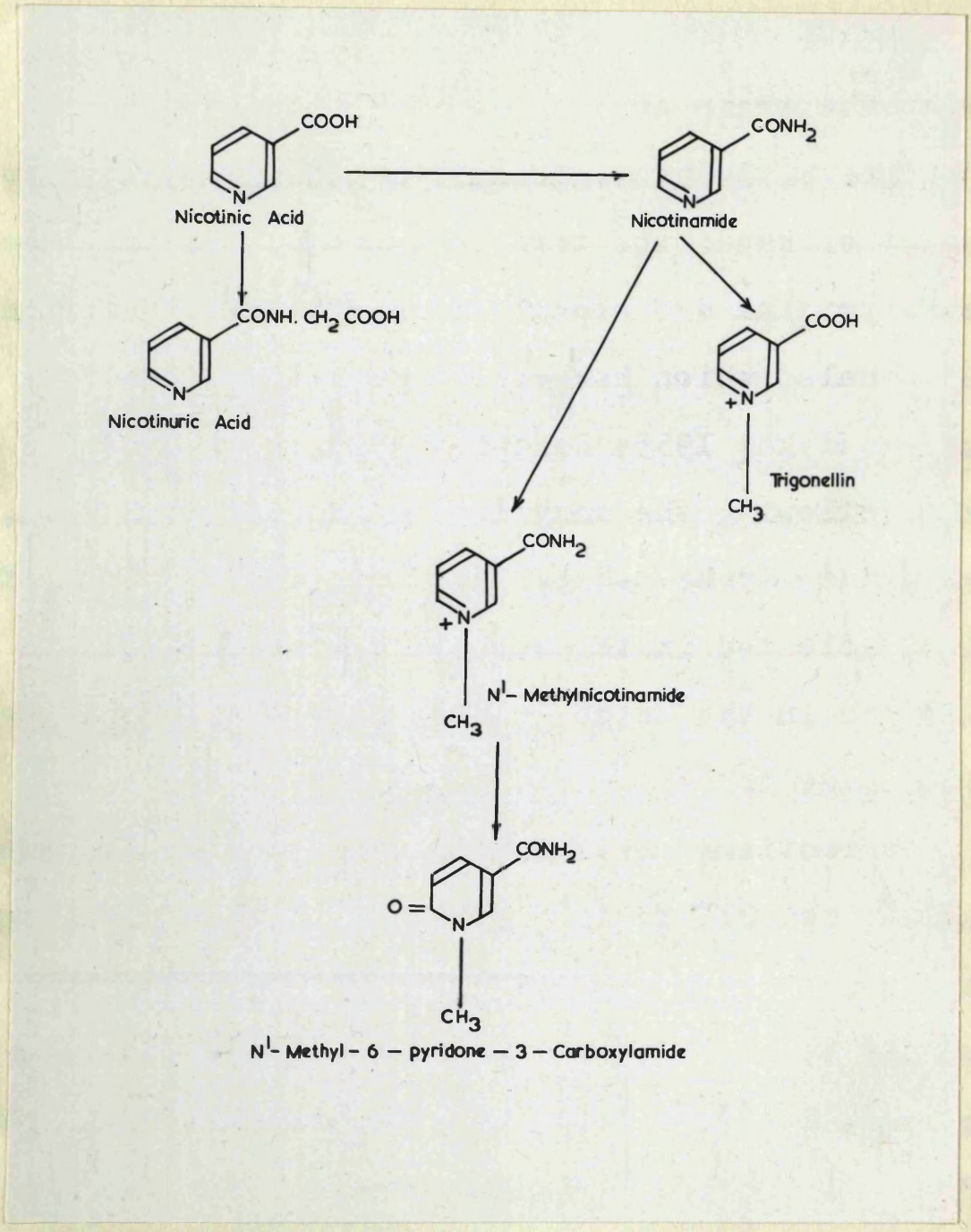

Fig. 8. Routes and Products of Metabolism of Nicotinic Acid and its Amide.

areas of the brain containing high concentrations of NADase (Hicks, 1955; Coggeshall & McLean, 1958). On the other hand, the toxic effects of 3-acetylpyridine are absent, or very much reduced in young animals, which have very low brain NADase activity (Hicks, 1955; Burton, 1957).

Following the enzymic cleavage of NAD, liberated nicotinamide may be metabolised by several routes. This is reflected in the various excretory products which occur in the urine (Singer & Kearney, 1954). These include trigonellin, nicotinuric acid and N'-methylnicotinamide (Fig. 8). While patterns of individual metabolites may vary in different species, generally, the N'-methyl derivative appears to be the major product of nicotinamide breakdown (Sarett, 1943; Ellinger & Coulson, 1944; Hundley & Bond, 1948; Leifer, Roth, Hogness & Corson, 1951; Chattopadhyay, Ghosh, Chattopadhyay & Banerjee, 1953). In some cases this compound may be further metabolised to the 6-pyridone derivative before excretion (Perlzweig & Huff, 1945; Knox & Grossman, 1946; 1947).

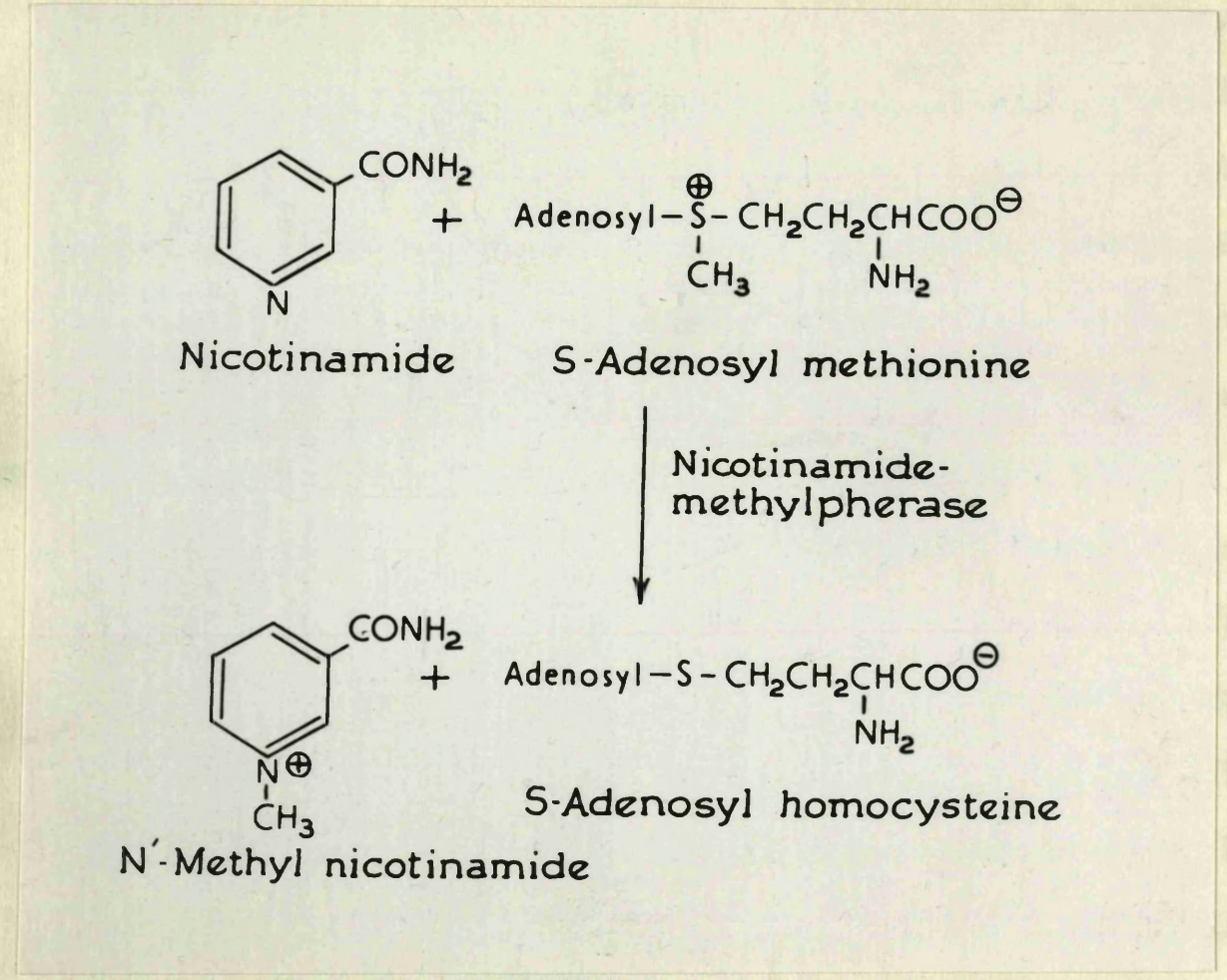

Fig. 9. Enzyme - Catalysed Methylation of Nicotinamide by an Active Derivative of Methionine.

The enzymic methylation of nicotinamide is accomplished by nicotinamide methylpherase, in the presence of methionine and ATP (Fig. 9). This complex reaction occurs as a result of the formation of an active intermediate, S-adenosyl methionine, which acts as a methyl donor (Baddiley, Michelson & Todd, 1949; Cantoni, 1951a,b).

The nicotinamide nucleotides are essential coenzymes in the main pathways of carbohydrate metabolism (Bueding & Farber, 1961; Bell, Davidson & Scarborough, 1965).

They occupy an important position in glycolysis, during which the oxidation of glucose to pyruvic acid provides two additional molecules of ATP (Fig. 10, Fig. 11). In this process NAD is reduced during the enzymic oxidation of glyceraldehyde-3-phosphate, as an essential preliminary stage before the energy-yielding conversion of 1,3-diphosphoglyceric acid to 3-phosphoglyceric acid (Fig. 10).

Since the total amount of NAD is limited, the reduced nucleotide is rapidly reoxidised to facilitate

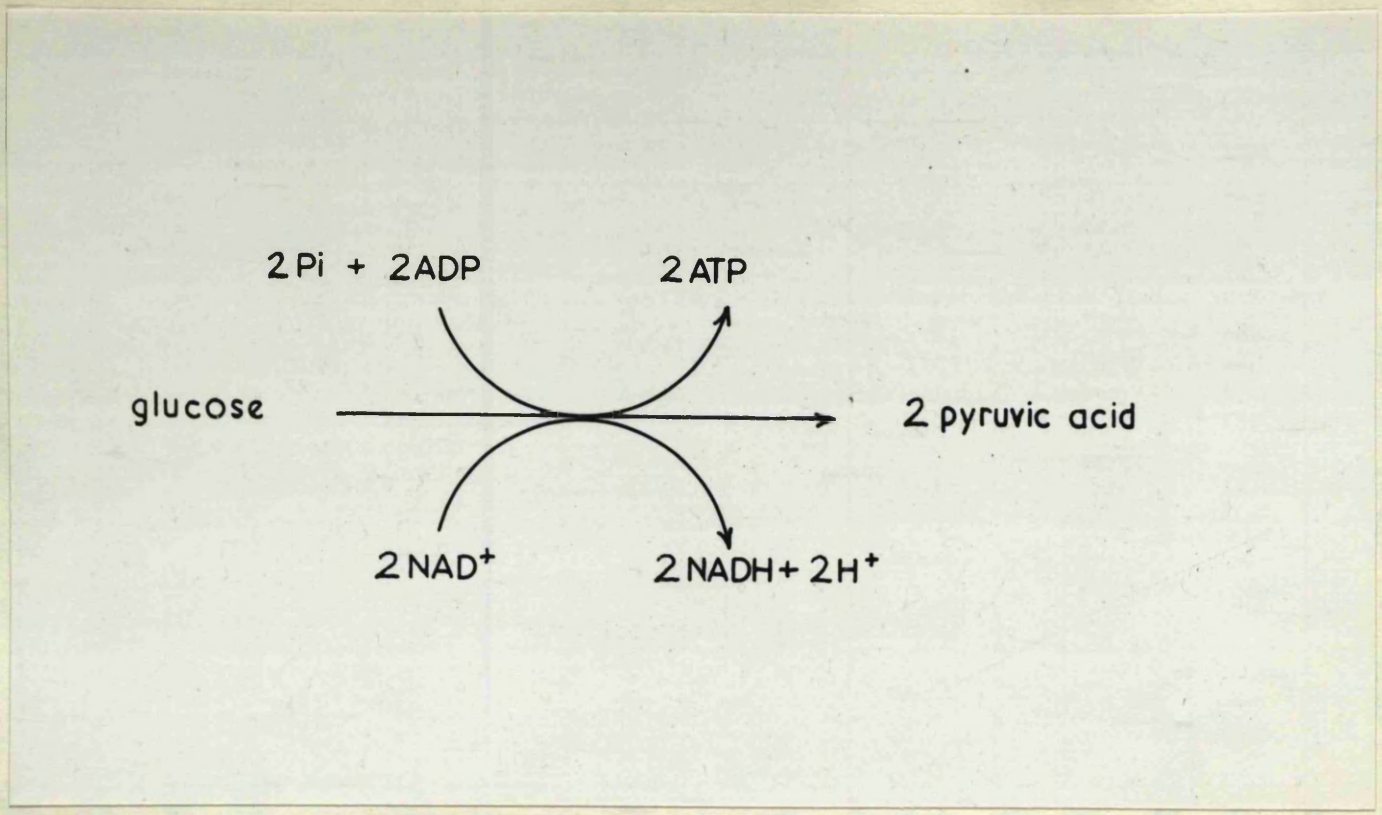

Fig. 11. Summary of Glycolysis, Showing the Synthesis of ATP and the Participation of NAD.

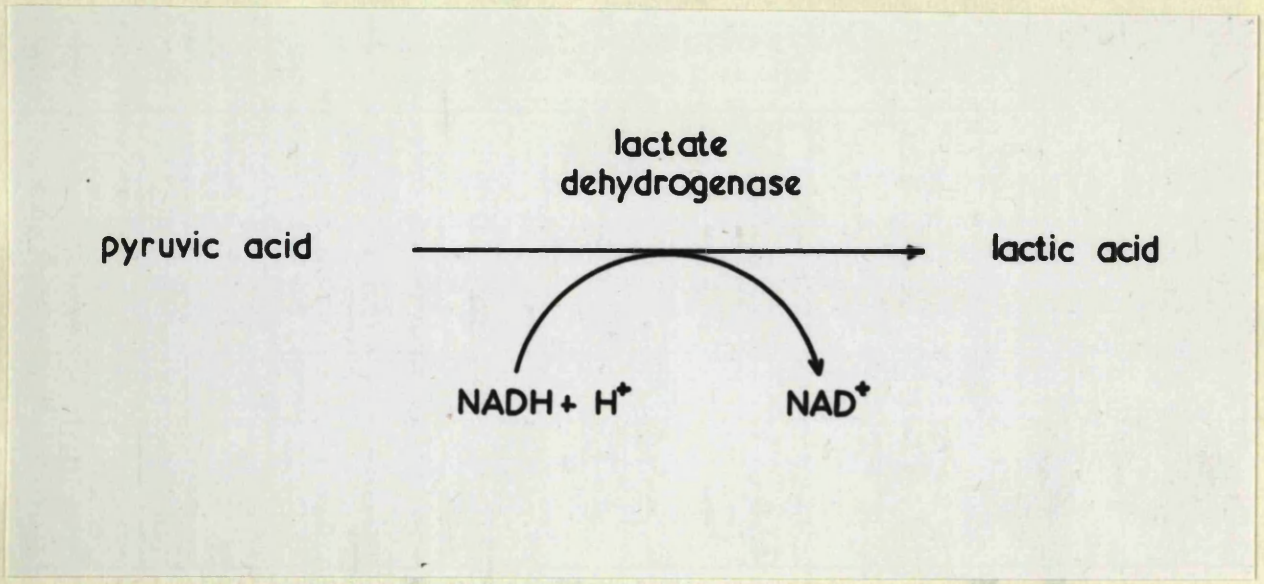

Fig. 12. Reoxidation of $NADH_2$ During the Conversion of Pyruvic to Lactic Acid.

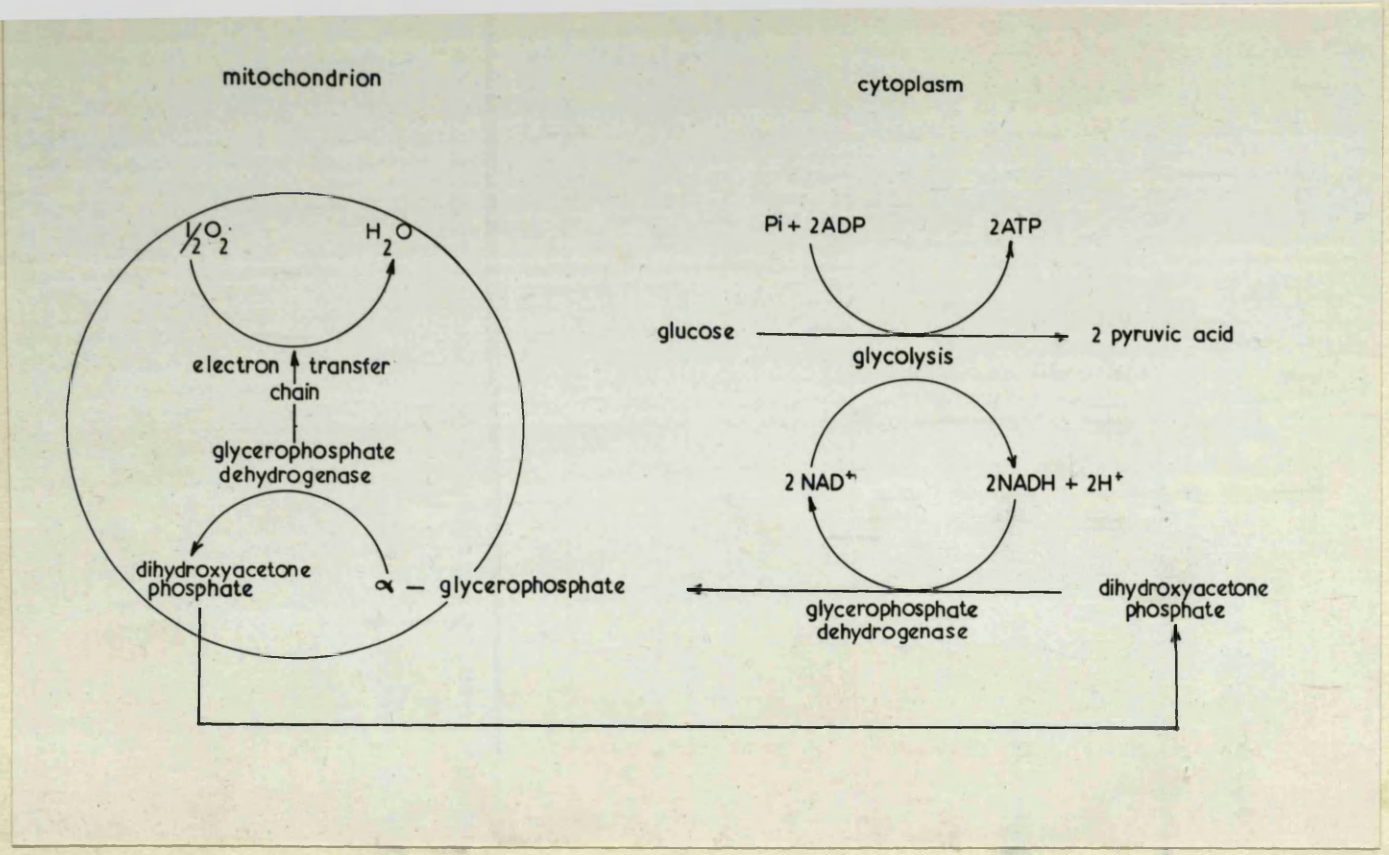

Fig. 13. "Shuttle System" for the Aerobic Reoxidation of $NADH_2$. Indirect Oxidation Through the Electron Transfer Chain is Dependent upon the Conversion of Dihydroxyacetone Phosphate to α-Glycerophosphate Within the Cytoplasm. Cyclic Reversal of this Reaction Subsequently Occurs Within the Mitochondria.

further glycolysis. This is accomplished in aerobic conditions through the electron-transfer chain (Fig. 15), which provides additional ATP. In anaerobic conditions $NADH_2$ is reoxidised during the conversion of pyruvic to lactic acid. This mechanism provides no additional ATP (Fig. 12).

Because of the impermeability of the mitochondrial membrane to nicotinamide coenzymes, cytoplasmic $NADH_2$, produced during aerobic glycolysis, cannot be directly oxidised by the electron transfer chain within the cristae of the mitochondria (Bell et al, 1965). Reoxidation therefore occurs by means of a number of so-called "shuttle systems", which link extramitochondrial nicotinamide coenzymes with the intramitochondrial respiratory chain (Lehninger, 1964). An example of this mechanism is the α-glycerophosphate-dihydroxyacetone phosphate system. In this case, $NADH_2$ is oxidised during the conversion of dihydroxyacetone phosphate to α-glycerophosphate, within the cytoplasm (Fig. 13). Following this reaction, α-glycerophosphate readily penetrates the mitochondria, where it is

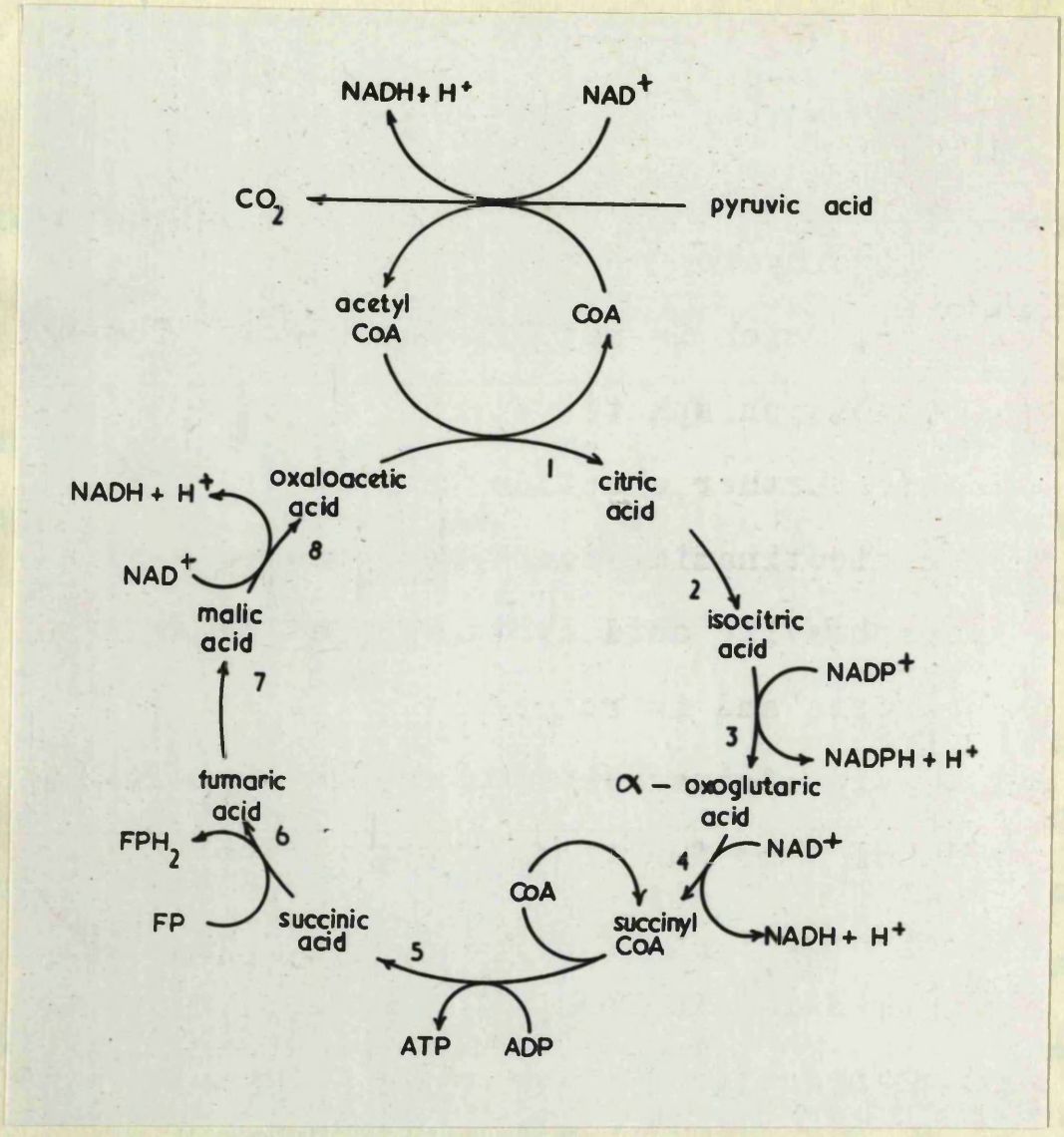

Fig. 14. Reactions of the Tricarboxylic Acid Cycle, Showing the Participation of the Nicotinamide Nucleotides in the Preliminary Acetylation of Coenzyme A and in Stages 3, 4 and 8 of the Cycle itself.

reoxidised to dihydroxyacetone phosphate by another dehydrogenase, which is not NAD-dependent. Thereafter dihydroxyacetone phosphate returns to the cytoplasm to facilitate further electron transfer.

The nicotinamide coenzymes also participate in the tricarboxylic acid cycle, which occurs within the mitochondria and is responsible for the oxidation of pyruvic acid, formed during aerobic glycolysis. As a result of this function, these nucleotides are therefore indirectly involved in the interconversion, syntheses and oxidation of fats and amino acids as well as carbohydrates (Singer et al, 1954).

Initially, NAD acts as an essential cofactor, together with α-lipoic acid and thiamine pyrophosphate, in the oxidative decarboxylation of pyruvic acid and acetylation of coenzyme A (Fig. 14) (Bell et al, 1965). $NADH_2$ generated during this complex preliminary reaction enters the respiratory chain while the acetyl-coenzyme A participates in the citric acid cycle (Lehninger, 1964). In the first stage of this sequence, oxaloacetic acid is converted to citric acid. Thereafter,

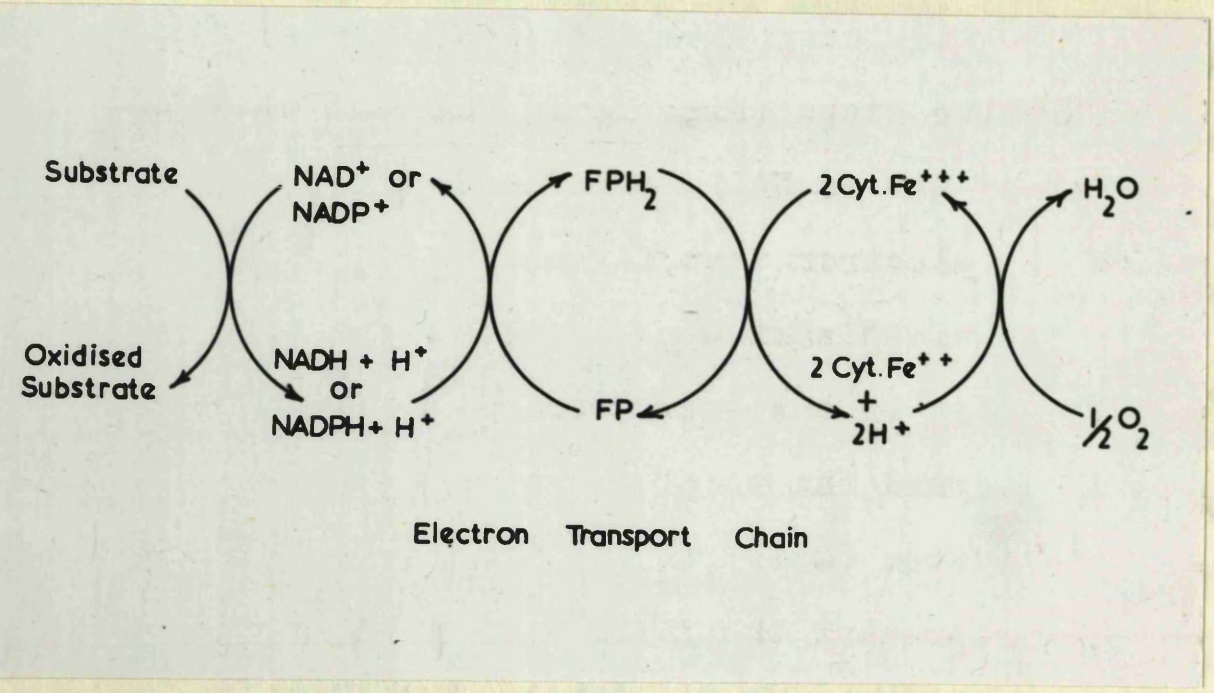

Fig. 15. Electron Transfer Chain Showing the Transfer of Electrons and H^+ from Substrate to Molecular Oxygen, Via the Nicotinamide Nucleotides and the Cytochromes.

the nicotinamide coenzymes catalyse three of the four oxidative steps (Fig. 14). In each case the reduction of NAD or NADP is followed by its reoxidation through the electron transfer chain.

The nicotinamide nucleotides also play an important part in the oxidation-reduction sequence, variously termed the respiratory chain or the electron transport system (Griffiths, 1965) (Fig. 15). These coenzymes represent the first link in this mechanism (Paul, 1964), which is the final stage of the major energy-yielding processes. The initial coenzyme reduction is followed by the transfer of electrons through the respiratory chain between substrate and molecular oxygen. However, not all substrates, oxidised by this route require the participation of the nicotinamide nucleotides. For example, the oxidation of succinate is linked directly to the reduction of the flavoproteins (Fig. 15, Fig. 16).

The importance of the electron transfer chain rests upon the very large free energy changes associated with these initial dehydrogenation reactions.

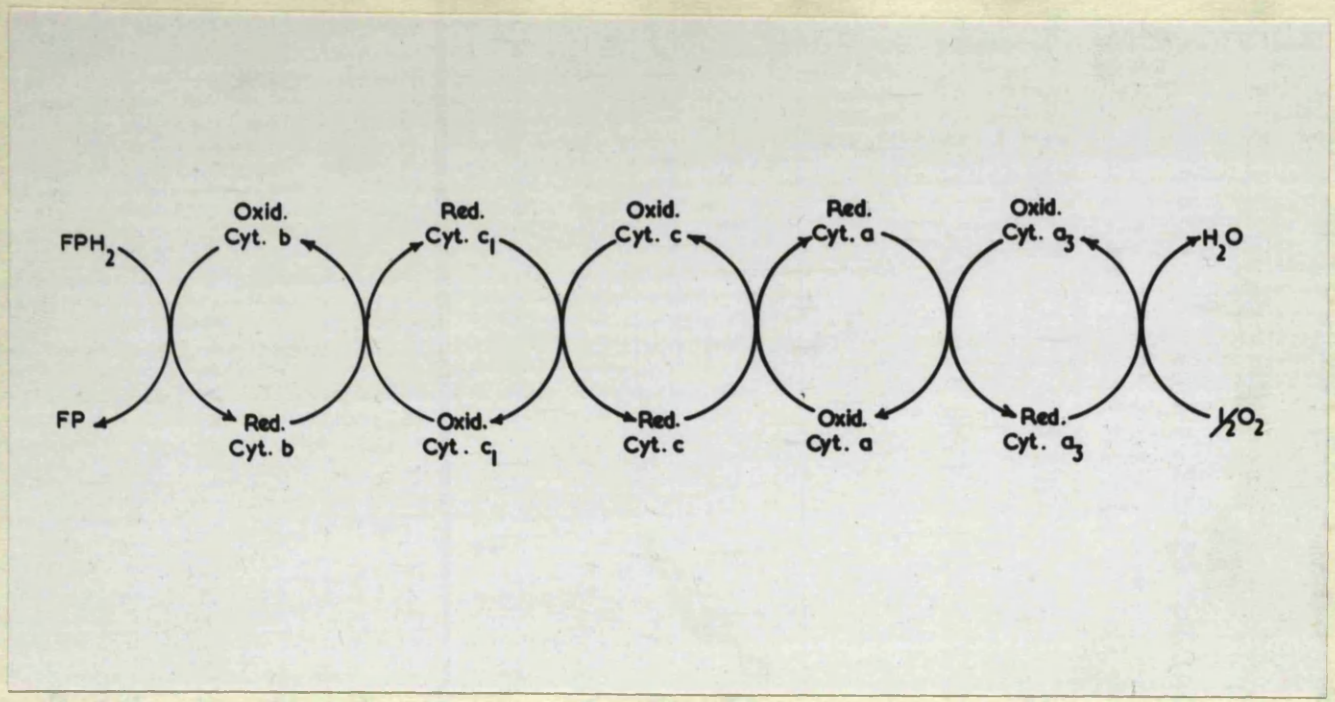

Fig. 16. Possible Sequence of Electron Transfer from the Flavoproteins Through the Cytochromes to Molecular Oxygen. Ubiquinone May also Participate Between Cytochrome b and Cytochrome c_1.

Throughout this process, free energy is conserved in the form of the phosphate bond energy of ATP. The coupling of the oxidation of fatty acids and the substrates of the citric acid cycle to the formation of ATP is therefore referred to as oxidative phosphorylation.

Although this pathway has been recognised for many years, the basic mechanisms involved in the various stages, have not been elucidated (Chance & Williams, 1956; Racker, 1961; Lehninger & Wadkins, 1962; Massey & Veeger, 1963). For example, the reactions concerning cytochrome b, the ubiquinones and cytochrome oxidase (cytochrome a_3) are still obscure and ill defined (Lester & Fleischer, 1959; Chance & Redfearn, 1961). Difficulty in understanding this process stems largely from the fact that these reactions occur within the insoluble multi-enzyme complex of the mitochondrion, which is less readily investigated than the cytoplasm.

The sites of phosphorylation within the respiratory chain have been localised (Chance & Hollunger, 1963). They occur between the nicotinamide

coenzymes and the flavoproteins, between cytochromes b and c, and between cytochrome c and molecular oxygen (Fig. 15, Fig. 16). However, doubt still surrounds the basic reaction mechanisms involved in these phosphorylations.

In this process, the ratio of phosphorus esterified to oxygen utilised is three, when the nicotinamide coenzymes are involved, but only two, when succinate is metabolised by this route. There is however, some thermodynamic evidence, that the oxido-reduction potential between the nicotinamide coenzymes and molecular oxygen is sufficient for the formation of over five molecules of ATP for each atom of oxygen reduced. This is supported by direct observations that P/O ratios may exceed three (Griffiths, 1965).

In oxidative phosphorylation, modification of the NAD molecule may occur other than by the introduction of hydrogen into the pyridine ring. However, no such modified NAD compounds have yet been identified and indeed, there is still no definite information concerning the precise chemical transformations

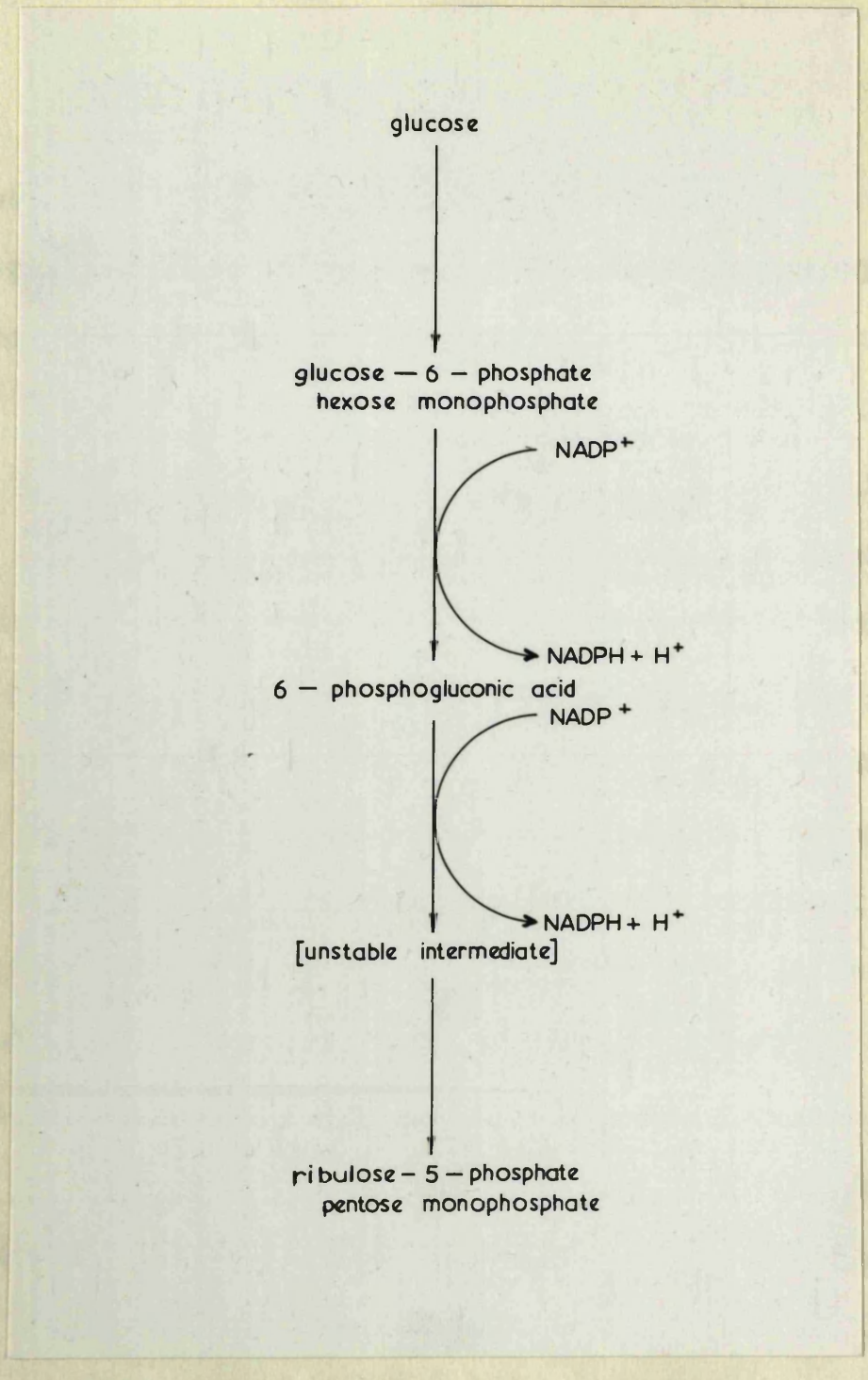

Fig. 17. Stages of the Pentose Phosphate Pathway, Showing the Participation of NADP in the Conversion of Hexose to Pentose Monophosphate.

involved. These hypothetical compounds may be related to the so-called "extra-DPN" of Purvis (1960), who demonstrated an alkali-stable form of NAD, which was unaffected by alcohol dehydrogenase. Such an alteration within the NAD molecule may consist of the addition and subsequent elimination of phosphate at either the 2 or 6 position of the pyridine ring (Kaplan, 1951; Burton & Kaplan, 1963). On the other hand, an additional phosphate group may be introduced into the adenine ring of NAD (Barltrop, Grubb & Hesp, 1963). Whatever the modification of the NAD molecule may be, it is possible that such a high energy derivative may have an important function in the phosphorylation of ADP (Pinchot, 1960; Griffiths, 1963).

The nicotinamide nucleotides play an important part in the first two stages of the pentose phosphate pathway, which is an alternative route for the oxidation of glucose (Fig. 17) (Greville, 1962). NADP is reduced during the dehydrogenation of glucose-6-phosphate to 6-phosphogluconic acid and also in the subsequent conversion of this compound to ribulose-5-phosphate.

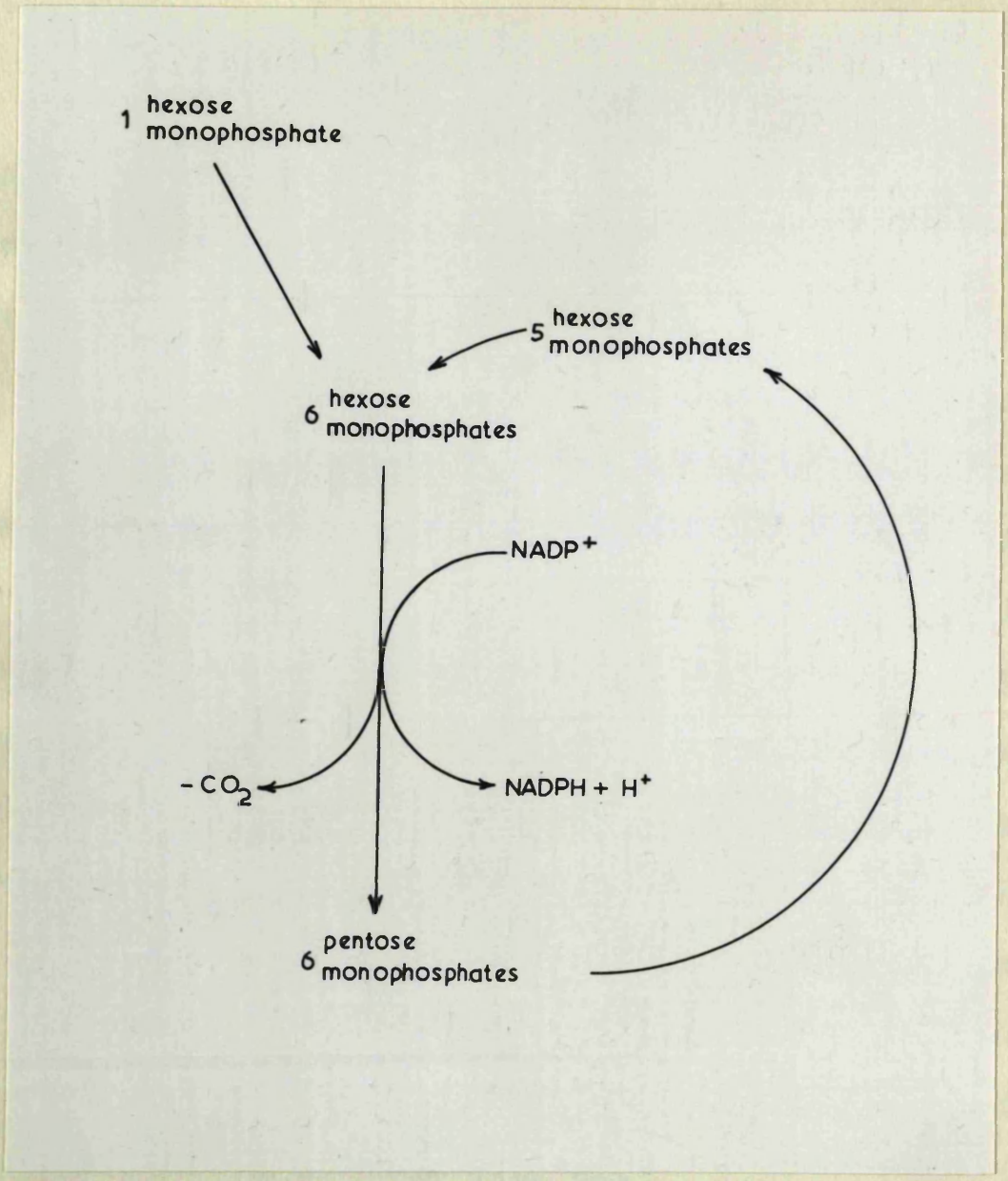

Fig. 18. Summary of the Role of NADP in the Pentose Phosphate Pathway, Showing the Cyclic Reconversion of Pentose to Hexose.

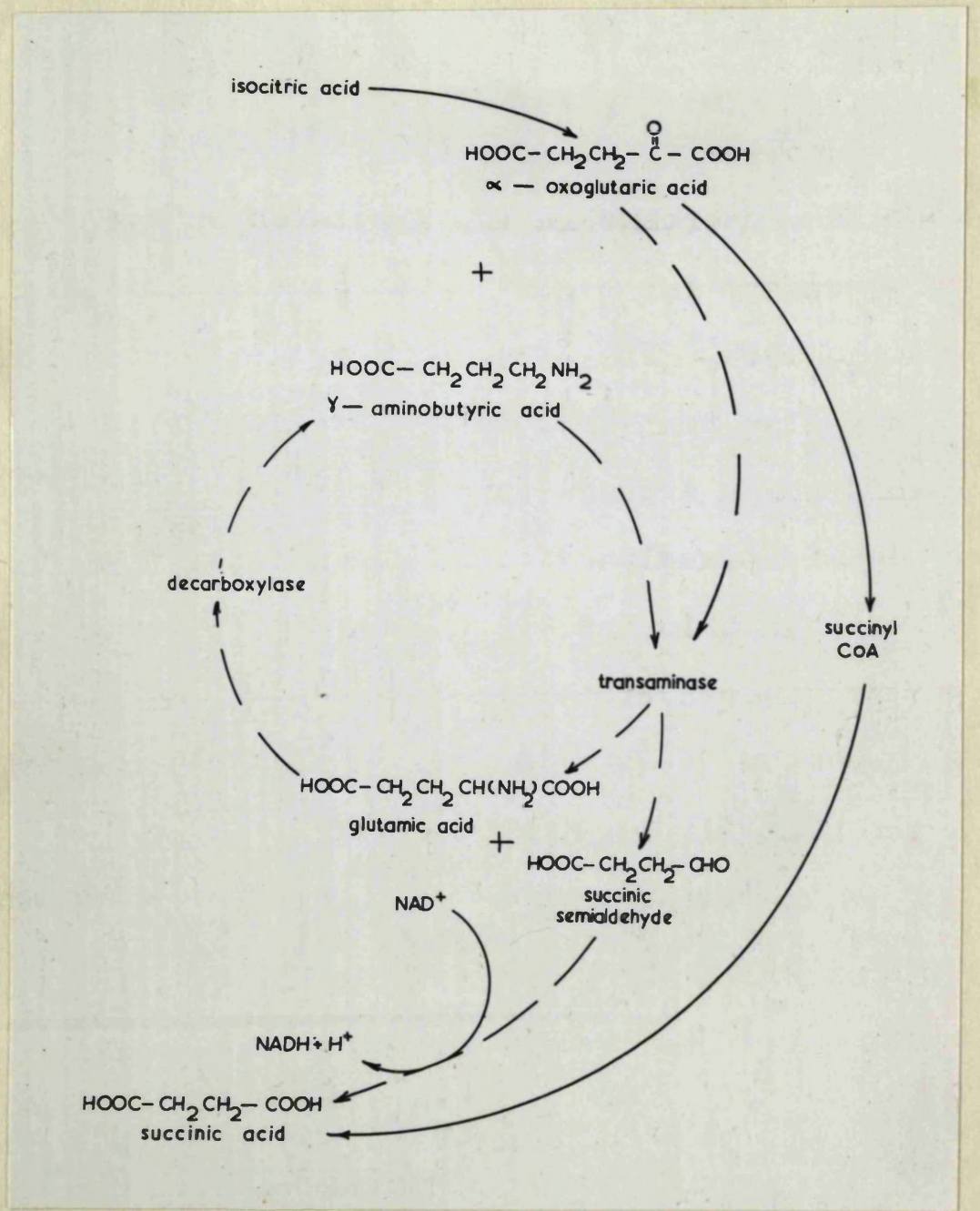

Fig. 19. Reactions of the GABA Shunt, Which Provides an Additional Route for the Metabolism of α-Oxoglutaric Acid to Succinic Acid.

The exact function of this route is not yet clear, but it may be important in the provision of the $NADPH_2$, necessary for certain synthetic reactions (Bell *et al*, 1965) (Fig. 18).

NAD also participates in the gamma-aminobutyric acid shunt, which acts as an additional route for the metabolism of α-oxoglutarate to succinate (Roberts, 1962) (Fig. 19). In this pathway, NAD is an essential coenzyme in the conversion of succinic semialdehyde to succinic acid (Fig. 19).

The nicotinamide nucleotides are involved also in several other reactions in cerebral metabolism. For example, NAD participates in the oxidation of glutamic acid to α-oxoglutaric acid (Fig. 20) (McIlwain, 1959), and NADP is an essential coenzyme in the reduction of the thiol group of the simple polypeptide, glutathione (Fig. 21). The metabolism of such substances as glutamic acid may only become important however, when there is a deficiency in the supply of the principal oxidative substrate, glucose (Tower, 1958; Quastel, 1962).

Although these various nicotinamide coenzyme-linked mechanisms are common to most tissues, slight differences occur in the relative importance of individual pathways in particular organs. Within the brain, there are present the various enzymes, cofactors and intermediaries necessary for glycolysis (Banga, Ochoa & Peters, 1939; Geiger, 1940; MacFarlane & Weil-Malherbe, 1941; Utter, Wood & Reiner, 1945; Elliott & Henry, 1946; Lowry, Roberts, Wu, Hixon & Crawford, 1954; Johnson, 1960), the tricarboxylic acid cycle (Krebs, Eggleston, Kleinzeller & Smyth, 1940; Abood, Gerard, Banks & Tschirgi, 1952; Srere, 1959; Winer, 1960; Vignais & Vignais, 1961), the pentose phosphate pathway (Dickens & Glock, 1952; Sable, 1952; Glock & McLean, 1954), oxidative phosphorylation (Ochoa, 1941; Case & McIlwain, 1951; Abood & Alexander, 1957; Berry & McMurray, 1957; Setchell, 1959) within the electron transfer chain (Brody, Wang & Bain, 1952; Gallagher & Buttery, 1959; Greville, 1962) and the gamma-aminobutyrate shunt (McKhann, Albers, Sokoloff, Mickelsen & Tower, 1960).

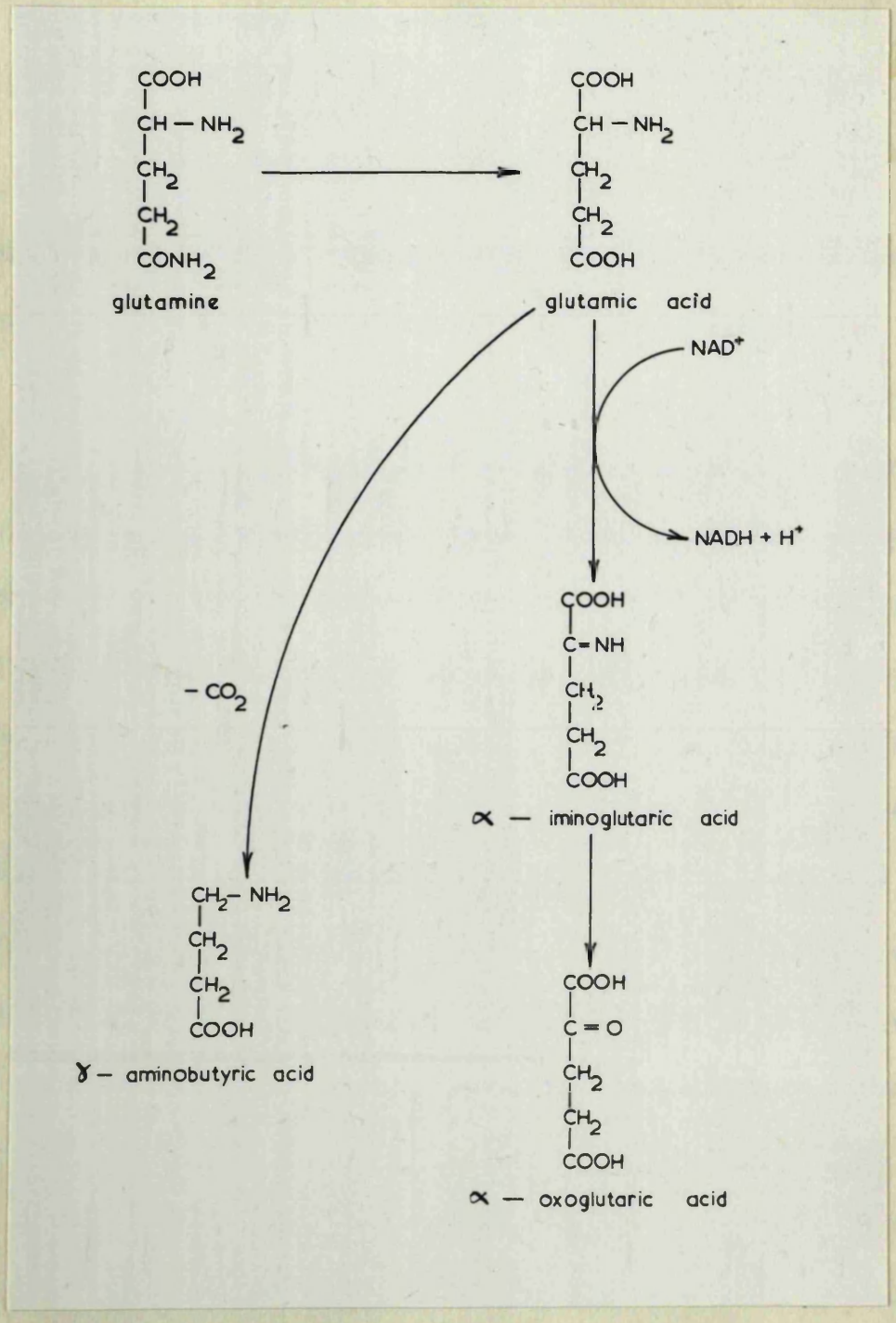

Fig. 20. Conversion of Glutamic Acid to α-Oxoglutaric Acid, Showing the Participation of NAD and the Parallel Synthesis of Gamma-Aminobutyric Acid.

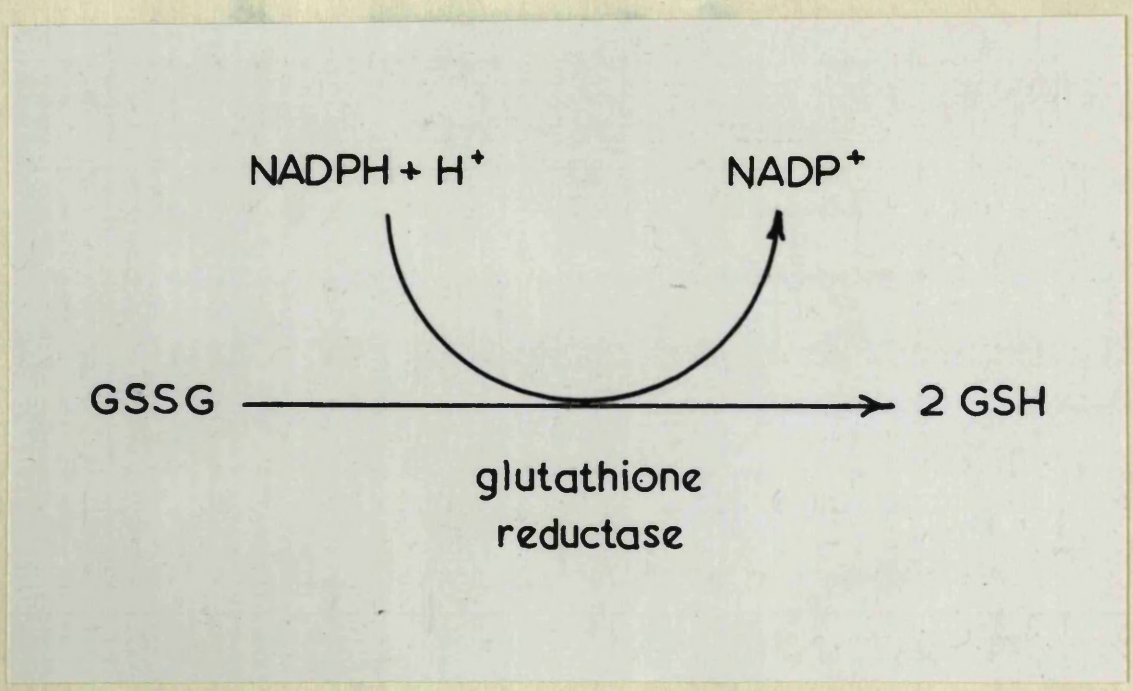

Fig. 21. The Relationship Between Glutathione Reduction and the Oxidation of NADP.

These metabolic pathways are not all equally important in brain energy metabolism. Neither do they all function continuously. There is indeed evidence that the pentose phosphate pathway is inoperative under normal conditions. This mechanism, or at least part of it may become active in certain abnormal circumstances (Hoskin, 1960).

The importance of the irreversible gamma-aminobutyric acid shunt in brain carbohydrate metabolism has not yet been clarified. It may represent the main oxidative pathway for α-oxoglutarate in some subcellular cerebral structures. On the other hand, its main functional significance may be ultimately associated with the participation of gamma-aminobutyric acid, either directly or indirectly, in the neurohumoral regulation of nerve cell polarisation (Roberts, 1956; Roberts & Baxter, 1958; Elliott, 1958).

The primary oxidative energy producing mechanism within the brain is the glycolytic pathway. This process utilises both glucose and to some extent, glycogen, which is degraded intracellularly by brain

phosphorylase. Following the partial oxidation of glucose to pyruvic acid (Fig. 10), metabolism mainly proceeds via the tricarboxylic acid cycle (Fig. 14). Some pyruvate is however converted to lactic acid (Fig. 12). During periods of increased central activity, glycolysis may be accelerated by a factor of twenty (McIlwain, 1959) and in these circumstances the ratio of lactate to pyruvate is very much higher than normal.

An examination of these pathways reveals a very important functional distinction between NAD and NADP. Whereas NAD is required mainly in the principal energy-yielding reactions, NADP is primarily concerned with the pentose phosphate pathway, which is only of minor importance in brain function. This operative demarcation between the nicotinamide nucleotides explains the preponderance of NAD in brain tissue.

The complex factors, which control and integrate the relative rates of glycolysis and oxidative phosphorylation are not yet understood. Nevertheless, certain correlations exist. There is, for example, an

inverse relationship between aerobic respiration and anaerobic glycolysis. Thus, in the phenomenon known as the Pasteur effect (Greville, 1962), increased respiration is associated with the inhibition of lactate formation. Conversely, in the Crabtree effect, excess glucose inhibits oxygen uptake. Attempts to explain these mechanisms rest mainly upon the basic assumption that competition occurs between metabolic pathways for essential common components. Such a competition may occur between the extra-mitochondrial glycolytic pathway and the mitochondrial respiratory chain for either ADP or inorganic phosphate (Lehninger, 1964).

The complexity of cerebral energy metabolism suggests that many factors may be involved in coordinating the various interconnected pathways. Since both the glycolytic and oxidative routes are reversible, it is likely that regulation of both the direction and velocity of a particular sequence of reactions may eventually depend upon the availability and oxido-reduction state of essential coenzymes.

The role of the nicotinamide nucleotides therefore seems to be of fundamental importance in the integration of energy metabolism as a whole, since NAD and NADP participate in so many of the basic energy-linked mechanisms.

Specific Introduction

Part II

The Relationship Between Nicotinic Acid, Nicotinamide Coenzyme Metabolism, Brain Function and Psychotropic Drug Action.

The possible relationship between mental abnormalities and the presence of certain toxic substances in the body has long been acknowledged (Osmond, 1960). However, controversy surrounds many of the attempts to identify the factors responsible (Leach & Heath, 1956; McGeer, McGeer & Boulding, 1956; Heath, Leach, Martens, Cohen & Feigley, 1960; Weil-Malherbe, Posner & Waldrop, 1962). Widespread interest has also been shown in the supposed similarities between certain psychotic states and the actions of such drugs as lysergic acid diethylamide, bufotenine, ibogaine, mescaline and psilocybin (Hoffer, Osmond & Smythies, 1954; Rinkel, Hyde & Solomon, 1954, 1955; Rinkel, Hyde, Solomon & Hoagland, 1955; Hoffer & Osmond, 1955; Fabing & Hawkins, 1956; Woolley & Shaw, 1957; Schneider & Sigg, 1957; Woolley, 1962). Despite their imperfect psychotomimetic properties (Lewin, 1931; Hollister, 1962; Curtis & Ryall, 1963), some of these drugs have been used to produce " model psychoses" (Fischer, 1954a,b) in order to facilitate the study of schizophrenia (Stockings, 1940). These, and other

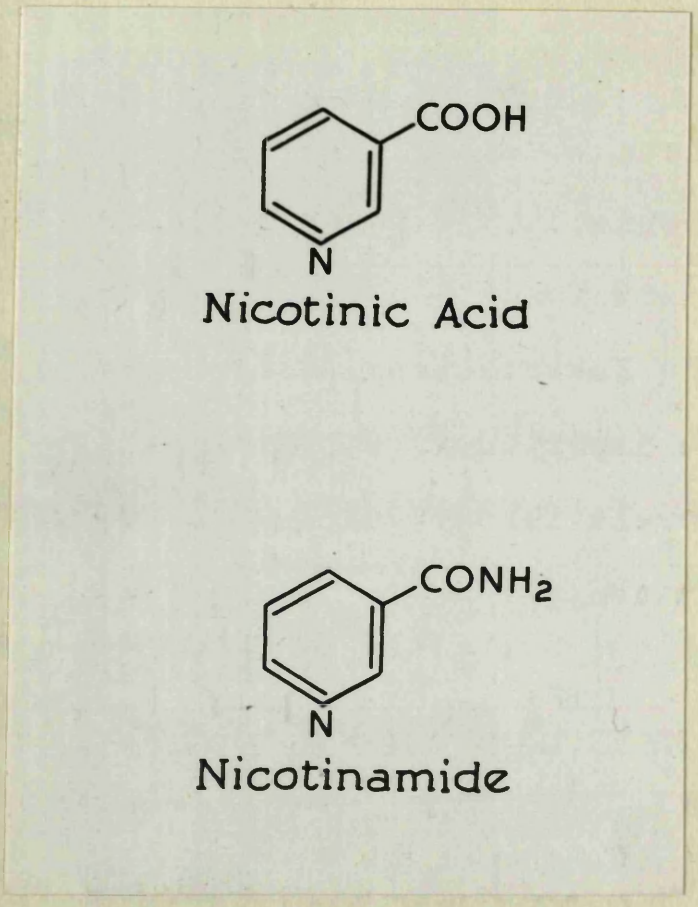

Fig. 22. Nicotinic Acid and its Amide.

recent investigations have largely overshadowed the discovery of the connection between pellagra psychoses and nicotinamide deficiency (Elvehjem, Madden, Strong & Woolley, 1937). Nevertheless, this relationship is of considerable importance, since it was one of the first positive correlations demonstrated between mental illness and the metabolism of a specific chemical compound.

The central effects associated with pellagra are both numerous and varied (Sourkes, 1962). Primary symptoms, consisting of insomnia, depression, confusion and increased irritability, often appear before peripheral ones (Hardwick, 1943; Leitner, 1948). Secondary signs, which represent exaggerations of the early symptoms, consist of delirium, hallucinations and stupor, leading eventually to irreversible psychotic conditions (Gregory, 1955). Many investigations have confirmed the efficacy of nicotinic acid and nicotinamide (Fig. 22) in the treatment of these central manifestations (Spies, Aring, Gelperin & Bean, 1938; Spies, Bean & Stone, 1938; Aring & Spies, 1939;

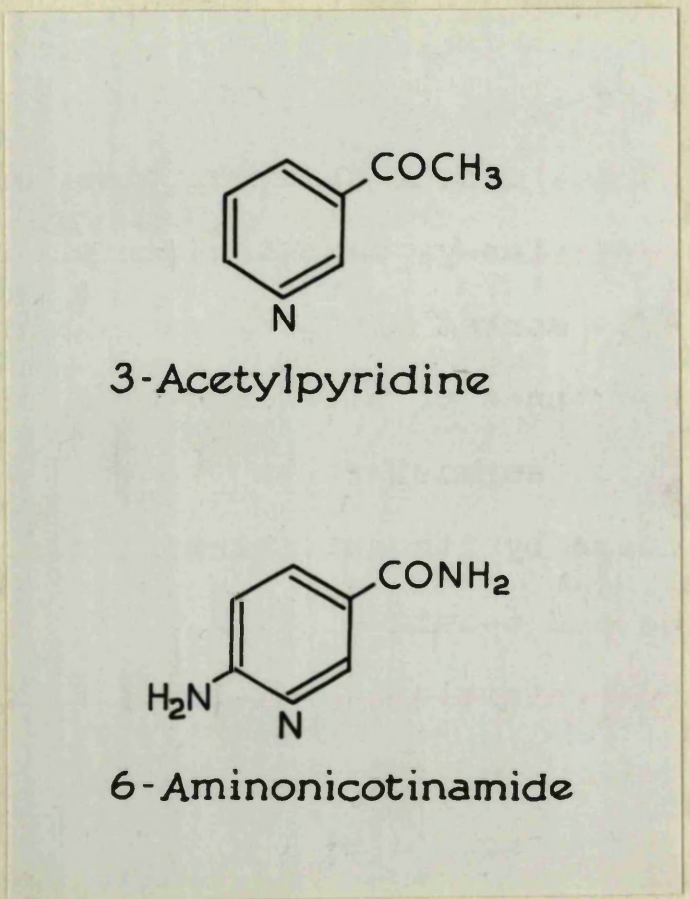

Fig. 23. Antimetabolites of Nicotinamide.

Wilkinson, 1944; Gregory, 1955; Sourkes, 1962). However, the extreme mental abnormalities, resulting from prolonged deficiency, cannot be reversed, since they correspond to marked histological damage (Leigh, 1952). The importance of nicotinic acid in brain functioning is also emphasised by the severe mental disturbances caused by its antimetabolites, 3-acetylpyridine and 6-aminonicotinamide (Fig. 23), which also produce histological lesions in the hippocampus (Woolley, Strong, Madden & Elvehjem, 1938; Woolley, 1945; Coggeshall & MacLean, 1958; Sternberg & Philips, 1959).

Nicotinic acid has been used to treat mental defects, apparently unrelated to pellagra. These include various confusional psychoses, depression and schizophrenia (Cleckley, Sydenstricker & Geeslin, 1939; Sydenstricker & Cleckley, 1941; Washburne, 1950; Gould, 1953; Hoffer et al, 1957). However, its value in these conditions has not been unequivocally accepted (Tonge, 1953; Ashby, Collins & Bassett, 1960). Nicotinic acid also diminishes the hallucinogenic

activities of lysergic acid diethylamide, when administered at the peak of the psychotomimetic experience. The complexity of this interaction is indicated by the psychotic effect, produced in some individuals, by prior treatment with nicotinic acid (Agnew et al, 1955).

Nicotinic acid produces a number of pharmacological effects, including peripheral vasodilation (Robertson, 1941; Williams, Eakin, Beerstecher & Shive, 1950) and an acetylcholine enhancing action (Williams et al, 1950). Nicotinamide has little vasodilating activity but reduces blood pressure, when administered in massive doses (Bergmann & Wislicki, 1953) and also produces marked hyperglycaemia (Feigelson, Williams & Elvehjem, 1951). However, the primary biological significance of these compounds rests upon their function as precursors of the nicotinamide nucleotides (Williams et al, 1950; McCollum, 1957; Harrow & Mazur, 1962) (Fig. 3), which are essential coenzymes in so many oxidation-reduction sequences (Kleiner & Orten, 1962). Large doses of

nicotinamide therefore increase liver, brain and spleen nicotinamide nucleotide levels (Kaplan, Goldin, Humphreys, Ciotti & Stolzenbach, 1956; Brown, 1964). Nicotinic acid has a similar effect on blood NAD (Hoagland, Ward & Shank, 1943; Burch, Storvick, Bicknell, Kung, Alejo, Everhart, Lowry, King & Bessey, 1955). It seems probable that the central actions of nicotinic acid and its amide will depend in some way upon modification of brain nicotinamide nucleotide metabolism.

Evidence suggesting a direct relationship between the nicotinamide nucleotides and altered mental function arises from investigations involving the major tranquillisers, reserpine and chlorpromazine (Burton, Kaplan, Goldin, Leitenberg, Humphreys & Sodd, 1958; Burton, Salvador, Goldin & Humphreys, 1960; Burton, Kaplan, Goldin, Leitenberg & Humphreys, 1960; Greengard & Quinn, 1962). These drugs maintain the nicotinamide-elevated NAD levels of liver throughout their period of tranquillisation. This response does not however occur in either blood (Burton, Salvador,

Smith & Howard, 1962) or brain (Brown, 1964). Chlorpromazine also modifies the activity of the enzyme, nicotinamide methylpherase (Burton & Salvador, 1962) (Fig. 9), by affecting the molecular conformation of the enzyme protein, with a consequent alteration in its affinity for nicotinamide. This relationship between the pharmacological effects of reserpine and nicotinamide metabolism is especially interesting since many investigations have associated reserpine tranquillisation with alterations in the brain levels of 5-hydroxytryptamine (Shore, Silver & Brodie, 1955; Pletscher, Shore & Brodie, 1955; Brodie, Pletscher & Shore, 1955; Brodie, Shore & Pletscher, 1956; Brodie, Tomich, Kuntzman & Shore, 1957; Shore, Pletscher, Tomich, Carlsson, Kuntzman & Brodie, 1957), which shares with nicotinamide the common precursor tryptophan (Harrow et al, 1962) (Fig. 5). Furthermore, nicotinic acid antagonises a number of the actions of 5-hydroxytryptamine (Woolley, 1958), which may have an important role in cerebral functioning (Woolley et al, 1954; 1957).

Hereditary errors of nicotinic acid metabolism may also affect mental performance. For instance, the biochemical defect responsible for Hartnup's disease occurs as a result of the close relationship between the biosynthetic pathways of 5-hydroxytryptamine and nicotinic acid (Fig. 5). This condition is characterised by the excretion of excessive amounts of 5-hydroxyindole acetic acid, a degradation product of 5-hydroxytryptamine, and subnormal amounts of kynurenine, a precursor of nicotinic acid. These changes are accompanied by psychoses, progressive mental deterioration and gradually increasing ataxia (Rodnight & McIlwain, 1955; Hersov, 1955; Evered, 1956 ; Baron, Dent, Harris, Hart & Jepson, 1956; Milne, Crawford, Girao & Loughridge, 1960; Musajo *et al*, 1964). Furthermore, a genetic disturbance of tryptophan metabolism may also be implicated in schizophrenia (Price, Brown & Peters, 1959; Brown, White & Kennedy, 1960; Benassi, Benassi, Allegri & Ballarin, 1961).

Another connection exists between nicotinic acid metabolism and mental abnormality. Thus, the

rate of nicotinamide oxidation in schizophrenics may be significantly higher than in normal individuals. The speed of this reaction is accelerated by hallucinogens and decreased by chlorpromazine (Heyman & Merlis, 1963; Heyman, 1964). It is not yet known whether this action of chlorpromazine is directly related to its therapeutic value as a tranquilliser. While this effect may be the result of a specific action upon a particular biochemical mechanism (Low, 1959a,b; Dawkins, Judah & Rees, 1959; 1960), it may also be a non-specific effect (Grenell, May, McElroy & Mendelson, 1959; Spirtes & Guth, 1961). Studies involving the mitochondrial enzyme $NADH_2$ - cytochrome C reductase, which is inhibited by chlorpromazine, support the latter view and suggest an action upon the structural organisation of the cell (Løvtrup, 1964).

Studies concerning the effects of drugs on oxidative phosphorylation and other biochemical mechanisms involving the nicotinamide coenzymes, have provided mainly inconclusive and contradictory results

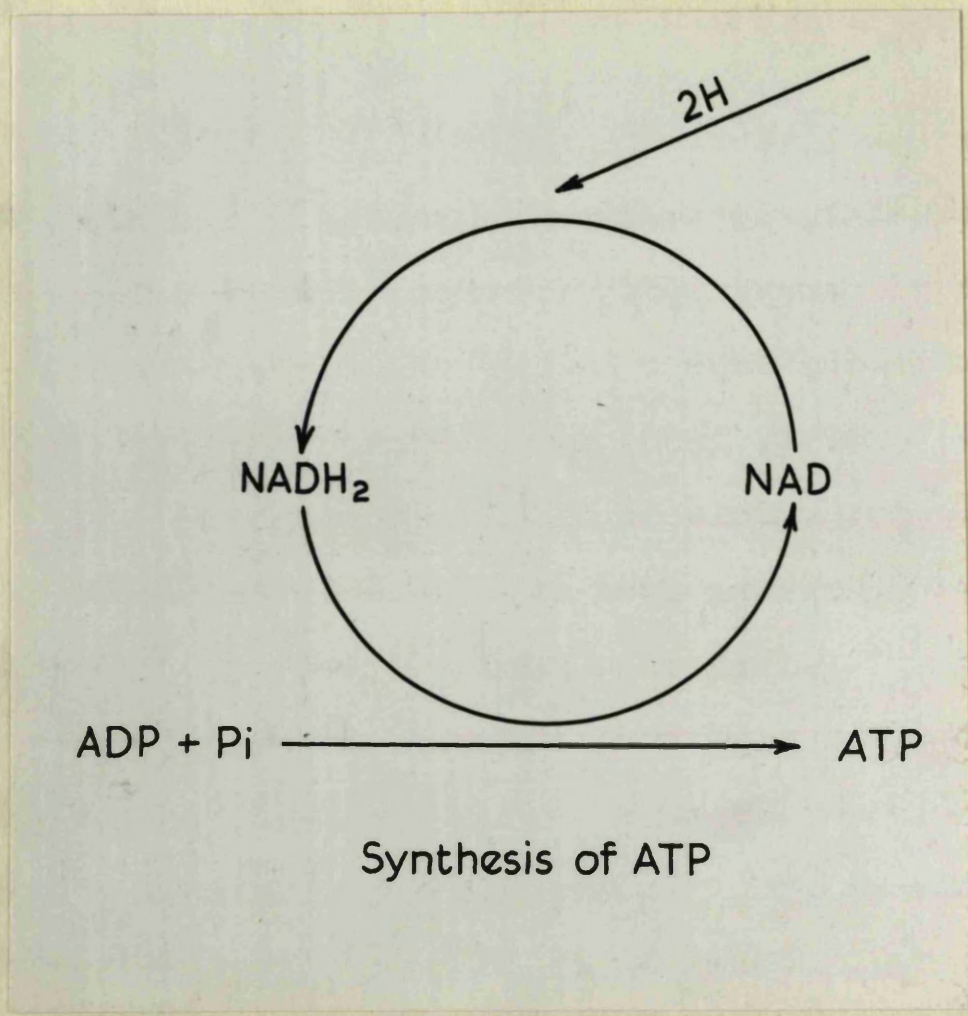

Fig. 24. The Relationship Between ATP Synthesis and the Oxidation of NAD.

(Mann & Quastel, 1940; Lu & Krantz, 1953; McIlwain, 1953; Ghosh & Quastel, 1954; Brody & Bain, 1954, Finkelstein, Spencer & Ridgeway, 1954; Fazekas, Albert & Alman, 1955; Frowein, Hirsch, Kayser & Krenkel, 1955; Kok, 1956; Century & Horwitt, 1956; Abood & Romanchek, 1957; Weiner, 1959). This is illustrated by the conflicting observations arising from studies on brain ATP levels, which are maintained as a result of the oxidation of reduced nicotinamide nucleotides in the electron transfer chain (McIlwain, 1957; Racker, 1961) (Fig. 24). Brain ATP levels may either fall (Kaul & Lewis, 1963a,b), rise (Grenell, Mendelson & McElroy, 1955), be unaffected (Weiner & Huls, 1961; Minard & Davis, 1962), or change in a complex manner (Kaul, Lewis & Livingstone, 1965) following tranquillising drugs. Antidepressives also produce contradictory results (Palladin, 1952; Palladin, Khaikina & Polyakova, 1952; Shapot, 1957; Bernsohn, Possley & Custod, 1959; Misson-Crighel, Constantinescu & Crighel, 1959; Van Petten & Lewis, 1962; Lewis & Van Petten, 1962; 1963). Generally however, behavioural stimulants, antidepressives and psychotomimetics

increase, whereas tranquillisers decrease brain ATP, with correspondingly opposite effects on ADP. These drugs have therefore been postulated to act by interfering with the fundamental mechanisms controlling the dynamic equilibrium between utilisation and resynthesis of ATP (Lewis et al, 1962; 1963; Kaul et al, 1963a,b; Lewis, Ritchie & Van Petten, 1965). In the case of the behavioural stimulants, this would increase the energy available for the syntheses of hypothetical neurohormones and the maintainance of ionic gradients within the brain (Richter et al, 1949; Richter, 1952; Kirshner, 1959; 1960; McIlwain, 1959; 1962; Heald, 1960; Humphrey & Coxon, 1963). The primary actions of behavioural stimulants seem therefore, to be upon synthetic pathways, since it is improbable that the increased neuronal activity, associated with central excitation, could be compatible with a decreased utilisation of ATP. This view is supported by the fact that in animals, rendered hyperactive by various physical means, brain ATP content falls (Le Page, 1946; Sytinsky, 1956). These results also suggest that the

reduction in brain ATP levels, produced by tranquillisers, may be due to an inhibition of the synthesis, rather than an increased utilisation of ATP.

It is unlikely that the chemically-diverse drugs in each group could produce similar effects, in an organ as complex as the brain, by a specific action on a single biochemical mechanism. Psychotropic drug-induced changes in brain ATP levels probably therefore reflect secondary responses, rather than primary pharmacodynamic mechanisms. Nevertheless, the possible importance of any modification of brain ATP cannot be overlooked, since this compound is of paramount importance in brain energy metabolism.

Nicotinamide nucleotide metabolism is also modified by the barbiturate, amylobarbitone. Experimental evidence indicates that this drug inhibits the oxidation of $NADH_2$ within the electron transport chain (Giuditta & Strecker, 1959). Such an interruption at the first stage of the respiratory chain inhibits both oxidative phosphorylation and metabolism through the tricarboxylic acid cycle, which

requires NAD initially for the acetylation of coenzyme A and subsequently for oxidations within the cycle itself (Fig. 14). This interference with ATP formation is reflected in the reduction of brain acetylcholine (Johnson & Quastel, 1953) and glutamine (Kini & Quastel, 1959) syntheses.

It is evident that the nicotinamide nucleotides play a very important part in cerebral energy metabolism. NAD in particular, is an essential coenzyme in all the main oxidative pathways, which supply ATP for neuronal activity. There is indeed evidence that NAD may participate indirectly, and possibly even directly, in the regulation and integration of such fundamental processes as oxidative phosphorylation and glycolysis.

The importance of these nucleotides in cerebral function is reflected in the severe mental and behavioural abnormalities resulting from dietary deficiency and hereditary defects in nicotinic acid biosynthesis. While the central manifestations of pellagra may be successfully treated with nicotinamide, there is no satisfactory evidence that this compound is

involved in any other form of cerebral malfunction.

Since a number of psychotropic drugs modify brain ATP levels, it is possible that this action might be associated with, or even dependent upon alterations in brain nicotinamide nucleotide metabolism. The likelihood of a relationship between psychotropic drug action and brain NAD metabolism is also suggested by the fact that the central depressant, amylobarbitone interferes specifically with the oxidation of $NADH_2$ within the respiratory chain.

Psychotropic drugs are widely used today. Yet, very little is known of their basic mechanisms of action or indeed, of the complex processes underlying abnormal or even normal brain functioning. This unsatisfactory and potentially dangerous situation, provides the mandate for pharmacological research in this field.

From the foregoing survey it is apparent that an investigation of the effects of psychotropic drugs upon brain nicotinamide nucleotide metabolism might provide valuable information concerning the fundamental pharmacodynamic activities of these drugs.

Purpose of Research

The work described in this thesis was undertaken to investigate the effects of a number of tranquillisers, antidepressives and psychotomimetics upon rat brain nicotinamide nucleotide levels. An attempt was made to determine how these drugs might modify brain nicotinamide nucleotide metabolism. In addition, the possibility of correlating alterations in the brain levels of these nucleotides with behavioural changes was also examined.

Psychotropic drugs are widely used today. Yet, very little is known of their basic mechanisms of action or indeed, of the complex processes underlying abnormal or even normal brain functioning. This unsatisfactory and potentially dangerous situation, provides the mandate for pharmacological research in this field.

From the foregoing survey it is apparent that an investigation of the effects of psychotropic drugs upon brain nicotinamide nucleotide metabolism might provide valuable information concerning the fundamental pharmacodynamic activities of these drugs.

The work described in this thesis was undertaken to investigate the effects of a number of tranquillisers, antidepressives and psychotomimetics upon rat brain nicotinamide nucleotide levels. An attempt was made to determine how these drugs might modify brain nicotinamide nucleotide metabolism. In addition, the possibility of correlating alterations in the brain levels of these nucleotides with behavioural changes was also examined.

Notes

1. The specifications of the chemicals and enzymes used in this investigation are described in Appendix I.

2. In this section the term "water" indicates the use of deionised water (conductivity $< 1.5 \times 10^{-5}$ ohms/cm), provided by either an Elgastat Type B 102 or a Type UB 110 deioniser.

3. All wavelengths reported in this section are uncorrected instrumental values.

4. Male Wistar rats (supplied by Messrs. A. Tuck & Son, Essex) were used throughout this study.

Methods

Notes

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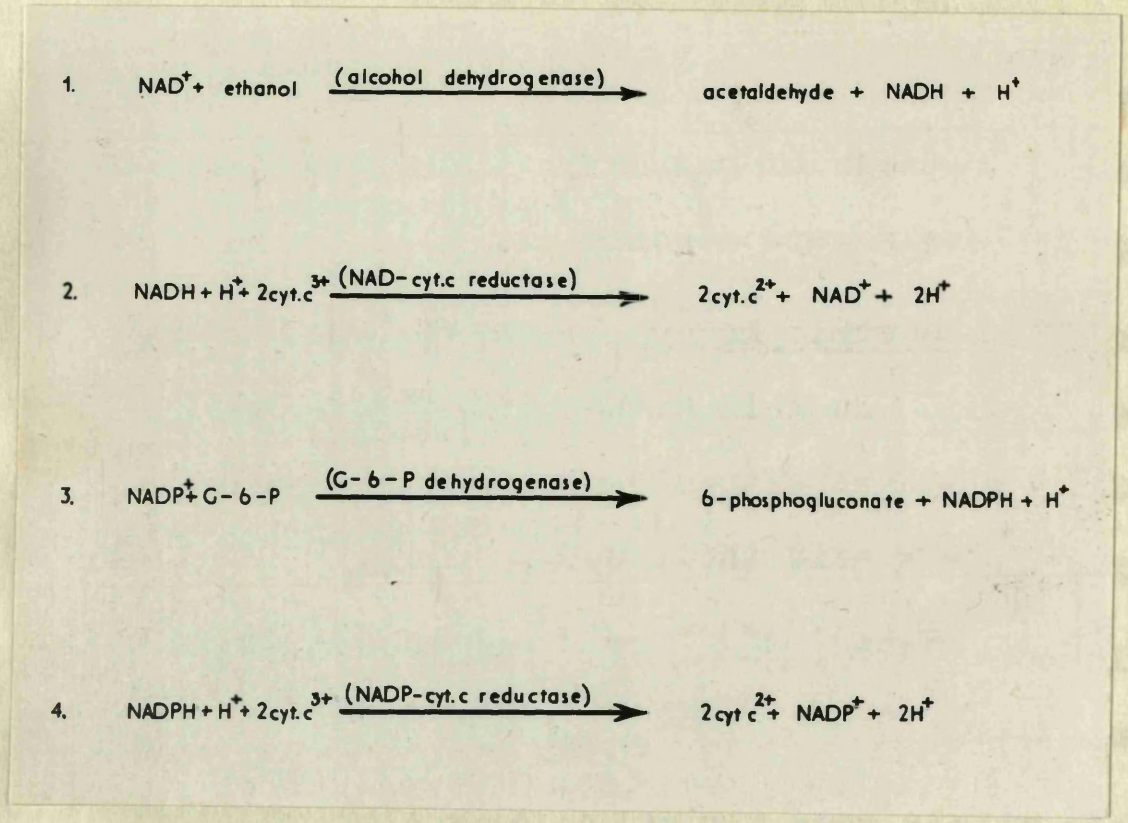

Fig. 25. Reactions Used in the Spectrophotometric Assay of the Nicotinamide Nucleotides.

Assay of Tissue Nicotinamide Nucleotides

Two methods of measuring tissue nicotinamide nucleotide levels were considered.

1. Spectrophotometric Assay

Introduction In this method (Glock & McLean, 1955) two separate tissue extracts are prepared using respectively hot acid (HCl, 0.1N, 90°C), which destroys the reduced nucleotides and hot alkali (NaOH, 0.1N, 90°C), which destroys the oxidised nucleotides (Schlenk, 1951). The assay depends upon measuring spectrophotometrically the rate of reduction of cytochrome c in the presence of neutralised tissue extract aliquots and the necessary accessory enzymes and substrates (Fig. 25). Thus $NADH_2$, continuously formed in the presence of ethanol and alcohol dehydrogenase (Fig. 25, reaction 1), and $NADPH_2$, produced during the interaction of glucose-6-phosphate and its dehydrogenase (Fig. 25, reaction 3), are coupled with their corresponding "cytochrome c reductases" (Fig. 25, reactions 2 and 4). These linked reactions (Fig. 25, 1 and 2; 3 and 4) therefore provide a means of

Enzyme Source	Activity (Units */mg)	Duration of Action (Days)
Bottom Ale Yeast	<2	-
	Nil	-
Bottom Lager Yeast	7	8
	<2	-
	Nil	-
Mixed Yeast	Nil	-
	9	6
	4	3

Table 1 Showing the Activity and Duration of Action of NADP - "Cytochrome c Reductase", Prepared from Different Types of Yeast (*Appendix IV).

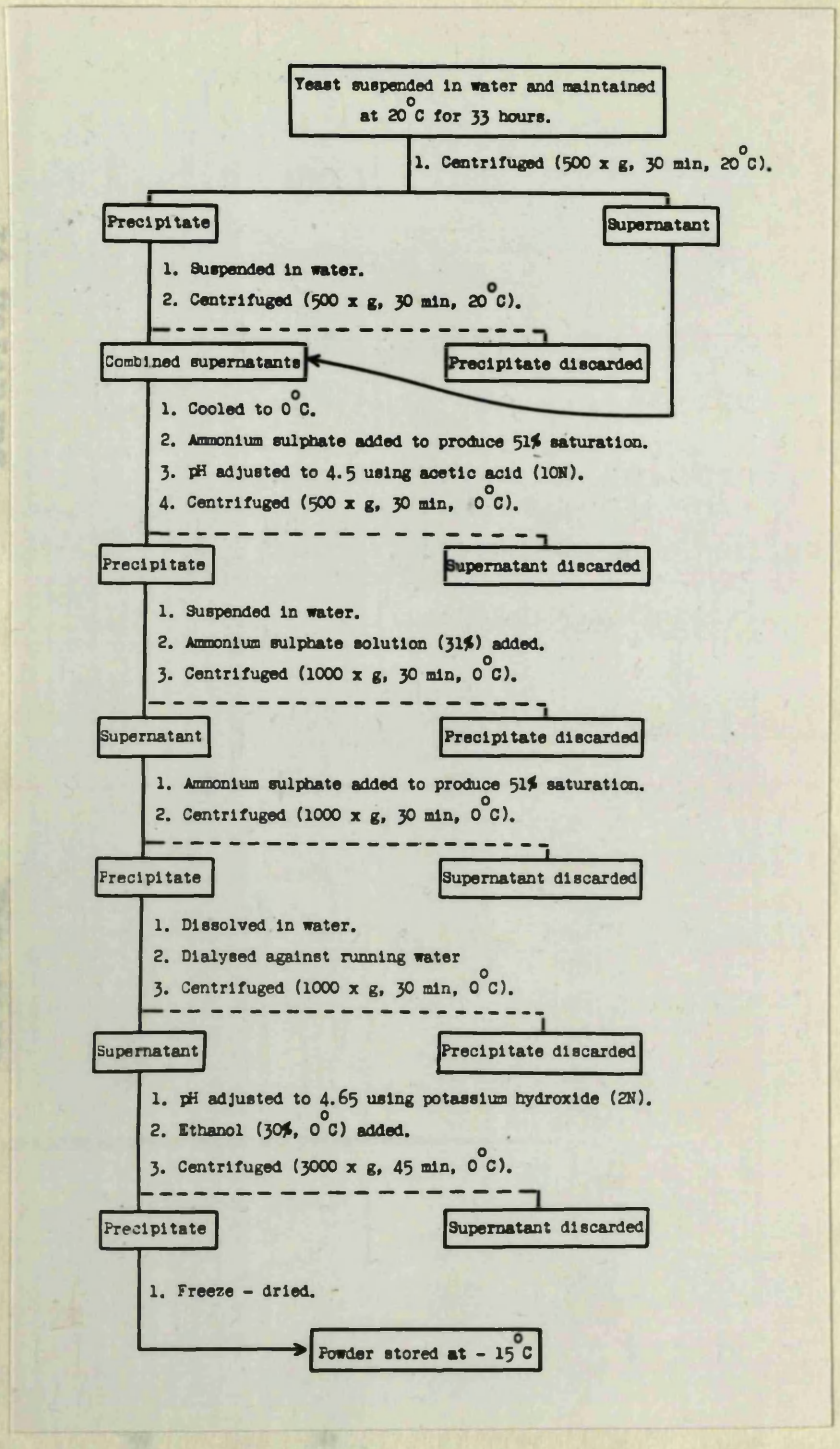

Fig. 26. Outline of the Stages in the Isolation of NADP - "Cytochrome c Reductase" from Yeast.

assaying tissue extract nicotinamide nucleotide levels, which influence the rate and amount of cytochrome c reduction occurring. The tissue nicotinamide nucleotide concentrations are finally calculated by comparing the increases in optical density at 550 mμ with those obtained in standard assay procedures using pure solutions of NAD and NADP.

Reason for Rejecting this Method Most of the enzymes and substrates required for this assay were commercially available. An exception was NADP - "cytochrome c reductase", which was isolated from yeast using the modified method of Haas, Horecker and Hogness (1940) (Fig. 26 and Appendix IV). However, because of the lability of this enzyme (Table 1) and the extreme difficulty experienced in preparing it, this method of assay was abandoned, since the estimation of NADP and $NADPH_2$ depended upon the availability of adequate supplies of NADP - "cytochrome c reductase".

2. Spectrophotofluorometric Assay

Introduction In this method, tissue nicotinamide nucleotides are extracted in hot (90°C) TRIS buffer

(0.05M, pH 8.2) and assayed fluorometrically (Lowry, Roberts & Kapphahn, 1957). The basis of this method is the destruction of the oxidised coenzymes by incubation (38°C, 1 hr) with sodium hydroxide (8N), which converts the nucleotides into stable fluorescent products (Kaplan, Colowick & Barnes, 1951; Lowry et al, 1957). This method was chosen because of its sensitivity (Udenfriend, 1962) and ready adaptability to the routine analysis of large numbers of brain extracts.

Killing of Animal and Extraction of the Pyridine Coenzymes

A standard procedure was adopted in preparing the extracts to avoid biasing the results by variations in technique. Each rat was first stunned by means of a light blow on the head and then killed by a more severe blow on the throat. After cutting the spinal cord immediately behind the skull and removing the covering skin and the top of the cranium, the whole brain was rapidly excised. This method facilitated the rapid removal and subsequent handling of the brain by minimising damage and intracranial bleeding. The brain was then

weighed on a torsion balance and transferred to TRIS buffer (15 ml, 0.05M, pH 8.2), previously heated (90°C) in a Potter-Elvehjem glass homogeniser (Fig. 27) (Greengard, Brink & Colowick, 1954). The time required for this whole operation varied between 25 and 35 sec. The tissue was disintegrated using a glass rod, and after 5 min heating (85°C), the homogeniser was cooled in an ice-bath for 20 min. The contents were then homogenised and the volume of the suspension adjusted to 20 ml using TRIS buffer (0.05M, pH 8.2, 0°C). The clear supernatant, obtained by centrifuging (40,000 xg, 0°C, 30 min) the homogenate, was decanted and stored at -15°C until required for the assay, which was always carried out within 2 days of preparing the extract.

Preliminary Investigation A number of studies were undertaken to determine the fluorescent characteristics of the NAD and NADP products and to find out whether this method was suitable for measuring brain nicotinamide nucleotides.

Determination of the Activating and Fluorescent Wavelengths of the NAD and NADP products The activating and

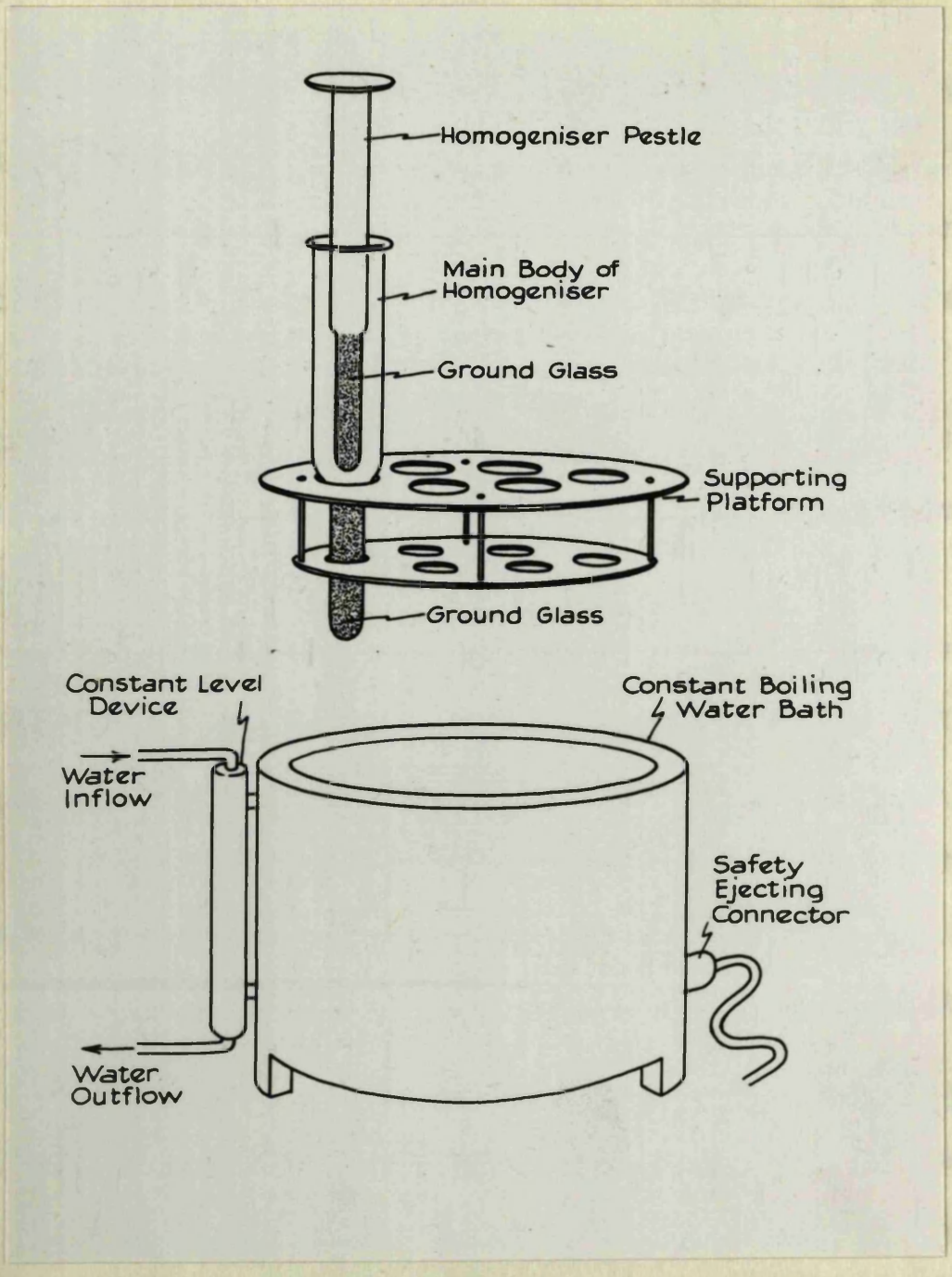

Fig. 27. Apparatus Used for Homogenising Rat Brains.

fluorescent wavelengths of the nucleotide products were determined by treating small quantities of NAD (0.003 μM in 0.5 ml of TRIS buffer, 0.05M, pH 8.2) and NADP (0.003 μM in 0.5 ml of TRIS buffer, 0.05M, pH 8.2) with 1.5 ml of sodium hydroxide (8N) for 1 hr at 38°C).

The NAD sample was then diluted to 8.0 ml with water and a 3.0 ml aliquot was transferred to a cell (1 cm) in an Aminco-Bowman spectrophotofluorometer. The photomultiplier shutter was locked in the open position (Fig. 28a) and the activating wavelength disc set at 220 mμ. The fluorescent wavelength was then scanned using the motor. At the end of each scan the activating wavelength was increased by 20 mμ and the sensitivity and meter multiplier controls adjusted to give satisfactory scale readings. This continued until the maximum reading was obtained. The fluorescent scan was then stopped and the fluorescent wavelength disc rotated manually to give a new maximum scale deflection. At this point the activating and fluorescent wavelengths, indicated by the appropriate discs, gave the activating and fluorescent wavelengths of the NAD product. A similar

procedure gave the wavelengths of the NADP product.

The activation spectra for the NAD and NADP products were determined by setting the fluoreseent wavelength disc to 465mμ (fluorescent wavelength of both the NAD and NADP products), rotating the activating wavelength disc in 10 mμ steps and noting the percentage transmission at each point (Fig. 28b). The fluorescent spectra were similarily obtained by adjusting the activating wavelength disc to 360 mμ and reading the percentage transmission at 10 mμ intervals throughout the fluorescent wavelength scale. Suitable TRIS buffer (0.05M, pH 8.2) blanks were prepared and examined to ensure that the fluorescent spectra obtained were authentic and not due to light scattering (Fig. 28b).

The activating and fluorescent wavelengths for both the NAD and NADP products were respectively 360 mμ and 465 mμ. These values agreed closely with those of Lowry, Roberts and Kapphahn (1957), who reported activating and fluorescent wavelengths of 360 mμ and 460 mμ respectively. The slight discrepancy between these results may have been due to instrument variation, for which no correction was made in either investigation.

Preparation of Work Curves for the NAD and NADP Products

Work curves were prepared to investigate the nature of the relationship between NAD and NADP concentrations and the fluorescence of their products. This procedure also indicated their minimum detectable concentrations.

A series of aqueous solutions of NAD was prepared. These contained respectively, 100 µg, 10 µg, 1.0 µg and 0.1 µg/ml of NAD. Aliquots (0.5 ml) were treated with sodium hydroxide (8N, 1hr, 38°C) before being diluted to 8 ml with water. Blank solutions containing only water were similarly treated. A sample (3 ml) from the most concentrated solution (100 µg/ml) was transferred to a cell (1 cm) in a spectrophotofluorometer. Having set the activating and fluorescent wavelengths to 360 mµ and 465 mµ respectively, the slit widths (Fig. 28a) were adjusted together with the sensitivity control to give satisfactory scale deflections (30 - 90%) at a meter multiplier setting of 1.0. The percentage transmission was then recorded. The fluorescence levels of the other samples were also measured using the same slit arrangements (Fig. 28a) (0, 3/16 in., 0:0, 3/16 in., 0:3/16 in.) and sensitivity setting (30). Suitable scale readings,

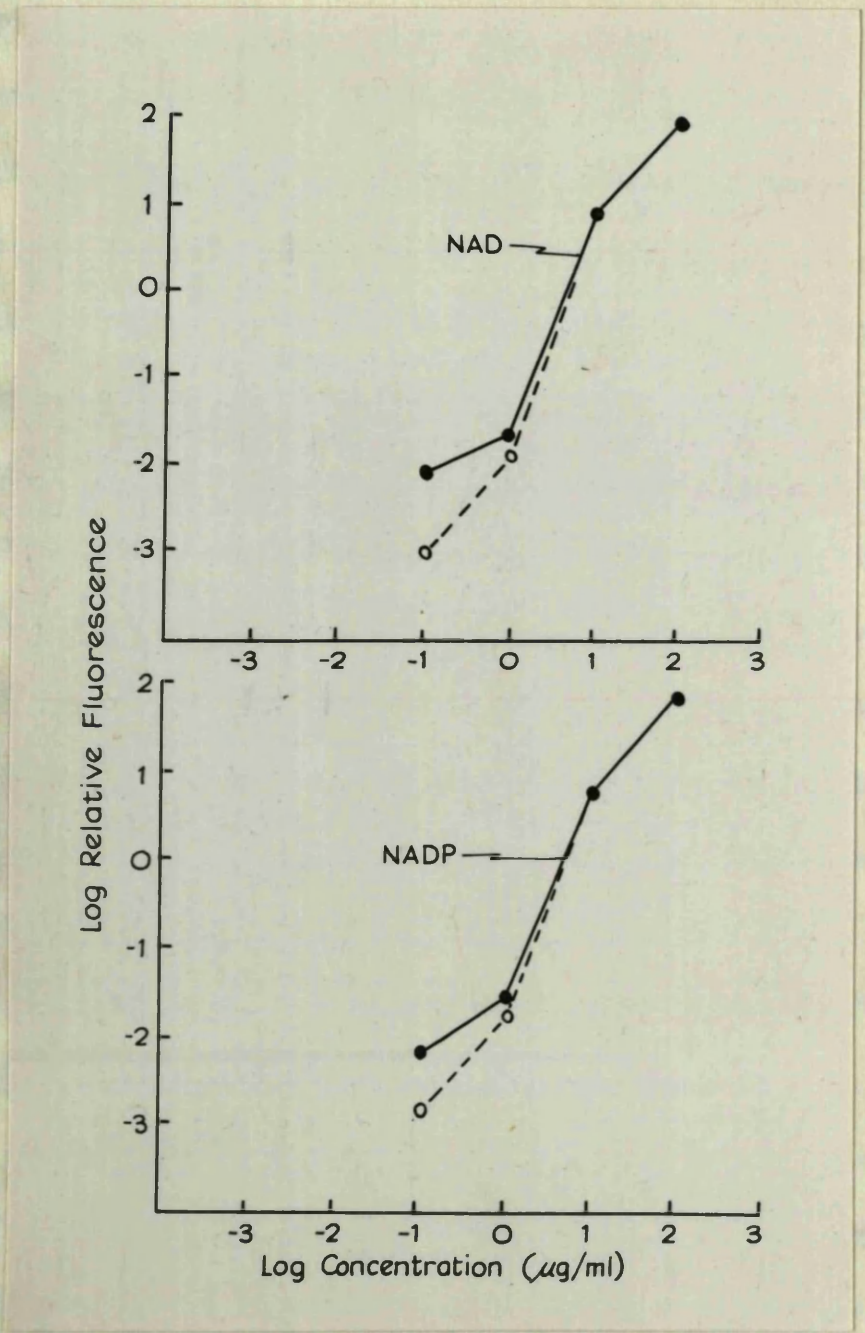

Fig. 29. Work Curves for NAD and NADP. The Broken Lines Indicate the Blank Subtracted Values.

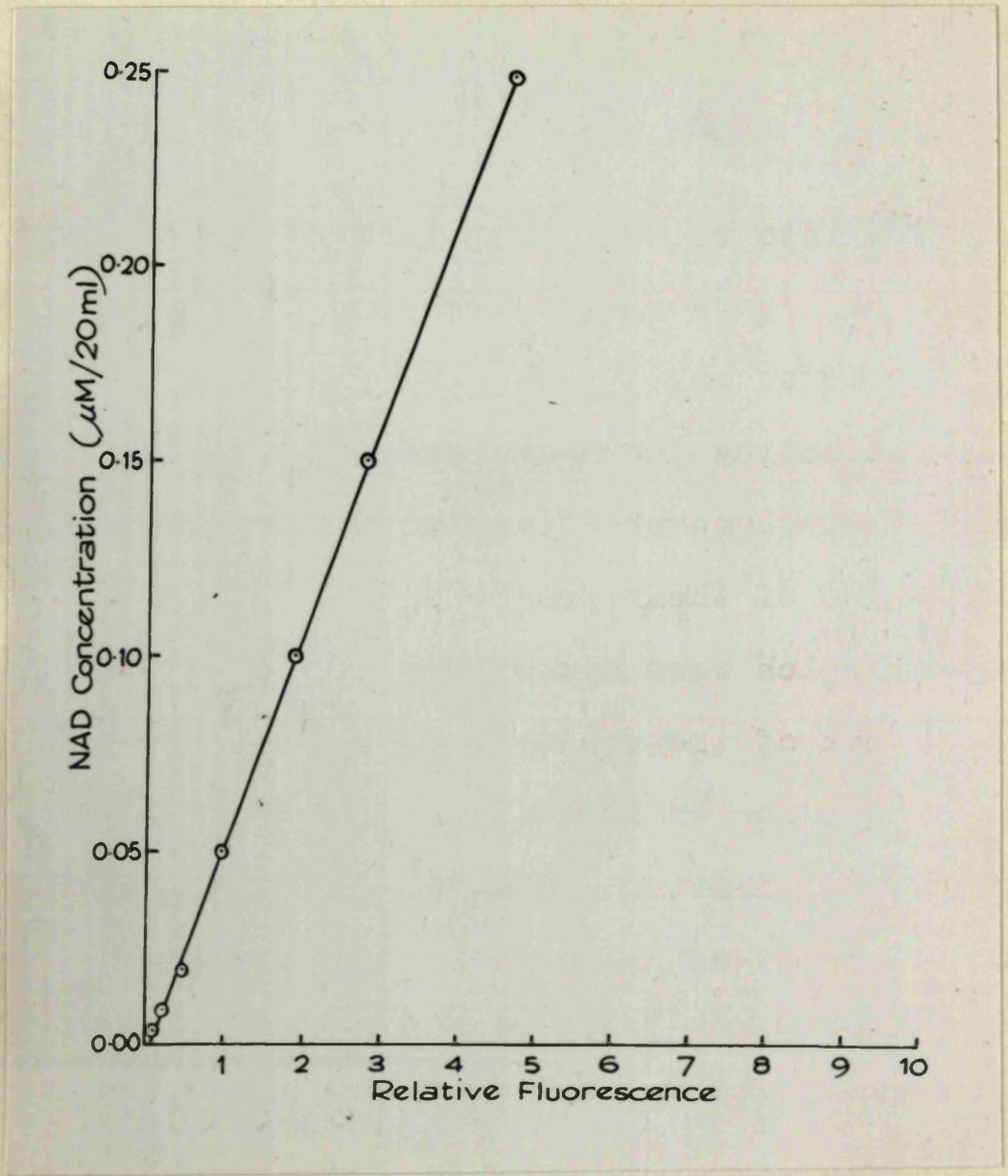

Fig. 30. Graph Showing the Linear Relationship Between NAD Concentration (μM/20 ml) and the Relative Fluorescence of a NaOH-Treated 0.5 ml Aliquot (Blank Subtracted).

obtained by altering only the meter multiplier control, were converted into relative fluorescence values by multiplying the percentage transmission by the corresponding meter multiplier setting. Work curves (Fig. 29), indicating the relationship between the logarithms of the concentrations of NAD and NADP and the fluorescence of their products, were then prepared. These curves, which were almost identical, showed that the fluorescence of the products did not vary linearly over the whole range of nucleotide concentrations. The curves showed flattening off at concentrations below 1µg/ml and quenching above 10 µg/ml.

However, examination of a further series of concentrations of NAD in the range likely to be found in brain extracts (Fig. 30) showed a linear relationship between nucleotide concentration and the fluorescence of the product. This experiment also indicated that the fluorometric method was sufficiently sensitive to permit the whole brain extract to be adjusted to 20 ml.

Examination of the Specificity of the Method The fluorescence was stable for several hours when maintained

Time (min)	% Transmission	Meter Multiplier	RF	% Transmission	Meter Multiplier	RF	% Destroyed
	NAD			NAD-Blank			
0	68.0	1.0	68.0	32.0	0.3	9.6	-
½	70.0	0.1	7.0	58.0	0.03	1.74	90.99
1	58.0	0.1	5.8	64.0	0.03	1.92	93.36
1½	55.0	0.1	5.5	55.0	0.03	1.65	93.41
3	50.0	0.1	5.0	45.0	0.03	1.35	93.75
	NADP			NADP-Blank			
0	40.0	1.0	40.0	52.0	0.1	5.2	-
½	72.0	0.03	2.16	31.0	0.03	0.93	96.46
1	55.0	0.03	1.65	27.0	0.03	0.81	97.59
1½	55.0	0.03	1.65	27.0	0.03	0.81	97.59
3	48.0	0.03	1.44	29.0	0.03	0.87	98.36
	NMN			NMN-Blank			
0	68.5	1.0	68.5	32.0	0.3	9.6	-
½	60.0	1.0	60.0	56.0	0.1	5.6	7.64
1	60.0	1.0	60.0	59.0	0.1	5.9	8.15
1½	58.0	1.0	58.0	48.0	0.1	4.8	9.67
3	61.5	1.0	61.5	20.0	0.3	6.0	5.77

Table 2. Showing the Effects of Incubating NADase with NAD (0.1 µM), NADP (0.1 µM) and NMN (0.1 µM) at 38°C and pH 4.2.

Brain Number	Relative Fluorescence		
	Untreated Aliquot	NaOH and HCl-Treated Aliquot (Blank)	HCl and NADase-Treated Aliquot
1	12.00	3.90	3.70
2	11.70	3.50	3.30
3	11.30	3.20	3.00
4	11.80	4.00	3.80
5	12.30	4.10	4.00

Table 3. Showing the Effects of Incubating Acidified TRIS Buffer Brain Extracts with NADase.

at a constant temperature but was not specific for NAD and NADP. Thus, strong alkali also converted nicotinamide mononucleotide (NMN) and nicotinamide riboside into highly fluorescent products. The reported absence of these two substances in brain tissue (McIlwain, 1957) was investigated by treating aliquots (2 ml) of a TRIS buffer (0.05M, pH 8.2) extract of rat brain with hydrochloric acid (120 µl, 5 min, 18°C) to destroy the $NADH_2$ and $NADPH_2$, and NADase (10 min, 38°C, pH 4.2), which destroyed NAD and NADP but not NMN (Table 2). The remaining fluorescence was then measured fluorometrically. This experiment demonstrated that neither NMN nor nicotinamide riboside was present in the brain extract (Table 3).

One advantage of this fluorometric assay was that neither N-methyl nicotinamide, the main excretory product of nicotinamide, nor nicotinamide itself, which was to be administered in subsequent experiments, fluoresced when treated with warm (38°C) alkali (8N) (Lowry et al, 1957).

Interfering Substances Fluorescent studies may be

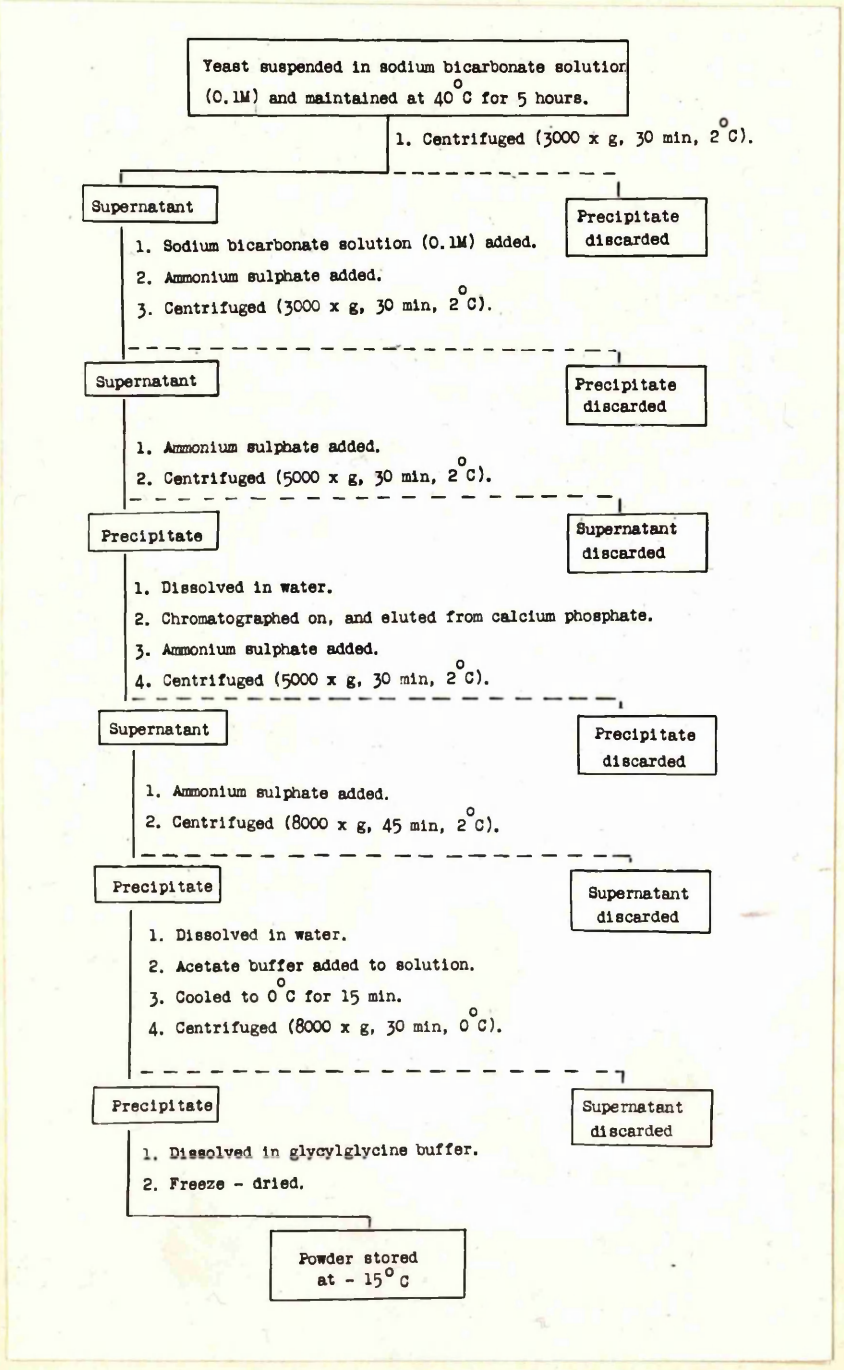

Fig. 31. Outline of the Stages in the Isolation of Glucose-6-Phosphate Dehydrogenase from Yeast.

affected by substances which prevent the development or interfere with the measurement of the fluorescent product. In this particular assay, carbonyl compounds including glucose, pyruvate, α-ketoglutarate and oxaloacetate, inhibit the development of fluorescence. Since some of the drugs to be investigated might have increased the brain levels of one or more of these compounds considerably, resulting in marked interference, separate internal standards were used for the drug and control extracts.

The possibility of interference from fluorescent materials other than the nicotinamide nucleotides was eliminated by using tissue blanks in which all the pyridine coenzymes had been destroyed by acid and alkali.

Availability of Accessory Enzymes The enzymes required for the preliminary stages of this method were readily available commercially. One of the enzymes, glucose-6-phosphate dehydrogenase, was occasionally prepared however from yeast (Kornberg, 1950) (Fig. 31) (Appendix IV).

Treatment of the Extract Aliquots for the Separate Determination of the Nicotinamide Coenzymes Aliquots (2 ml)

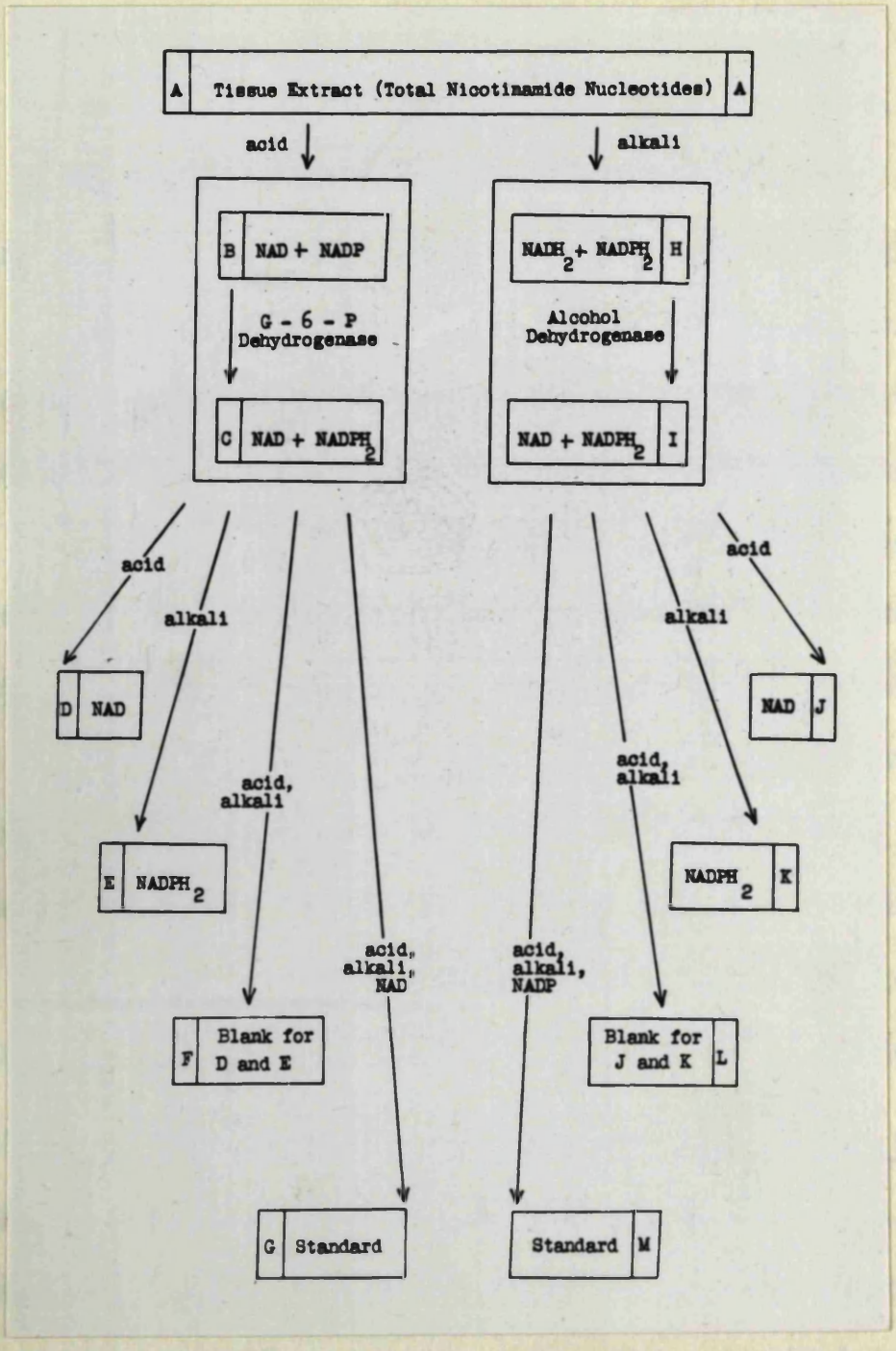

Fig. 32. Outline of the Stages in the Separation and Estimation of Tissue Nicotinamide Nucleotides.

of the extract were transferred to 2 glass test tubes labelled respectively "B/C" and "H/I" (Fig. 32). Sample B/C was treated for 5 min at 18°C with hydrochloric acid (120 µl, N) to destroy the reduced forms of the nucleotides. The pH was then readjusted to 8.2 using sodium hydroxide (120 µl, N). Sample B/C was incubated with 48 µl of glucose-6-phosphate (50 mM) and 30 µl of glucose-6-phosphate dehydrogenase for 30 min at 18°C to convert the NADP to $NADPH_2$ (Fig. 32).

Four aliquots (0.5 ml) of sample B/C were then transferred to test tubes labelled respectively "D", "E", "F" and "G" (Fig.32). Sample D was treated with 34 µl of hydrochloric acid (N) for 5 min at 18°C to destroy the $NADPH_2$. Sample E was incubated with 34 µl of sodium hydroxide (N) for 15 min at 60°C to destroy the NAD. Next, samples F and G were treated with 34 µl of hydrochloric acid (N) for 5 min at 18°C and then 34 µl of sodium hydroxide (2N) at 60°C for 15 min to destroy all the remaining nicotinamide nucleotides. In the fluorescence assay, sample F acted as a tissue blank and sample G, readjusted to pH 8.2 by adding 34 µl of hydrochloric acid (N) and modified by adding

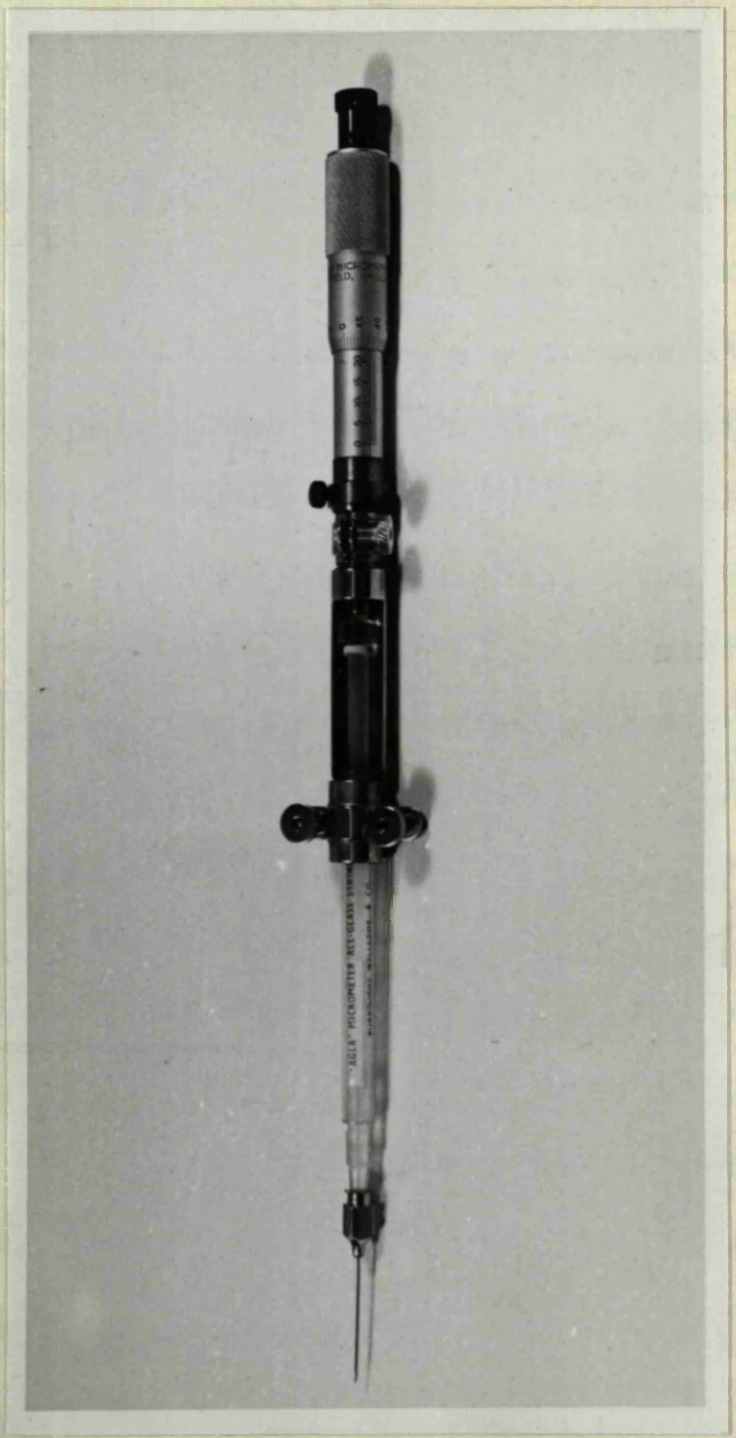

Fig. 33. Agla Micrometer Syringe.

NAD, was used as an internal standard. Throughout this assay small volumes were measured using an Agla micrometer syringe (Fig. 33).

Sample H/I was treated with 120 µl of sodium hydroxide (N) for 15 min at 60°C to destroy the oxidised forms of the nucleotides. The pH was readjusted to 8.2 with hydrochloric acid (120 µl, N). The sample was then incubated for 30 min at 180°C with acetaldehyde (32 µl, 2% v/v), alcohol dehydrogenase (30 µl) and EDTA (30 µl, 35 mM) to oxidise the $NADH_2$ to NAD (Fig. 32).

Four aliquots (0.5 ml) labelled "J", "K", "L" and "M" were treated with acid and alkali in the same manner as samples D, E, F and G respectively. In this case L was the blank and M, modified by adding NADP, acted as the internal standard.

All the samples (D, E, F, G, J, K, L and M) were finally treated with 1.5 ml of a solution containing sodium hydroxide (8N) and hydrogen peroxide (0.02%) for 1 hr at 38°C in a light-protected water bath. Each sample was then diluted to 8.0 ml with water and a 3.0 ml aliquot transferred to a cell (1 cm) in an Aminco-Bowman spectrophotofluorometer. Percentage transmission

readings, obtained at activating and fluorescent wavelengths of 360 mμ and 465 mμ respectively, were converted into relative fluorescence values by multiplying by the meter multiplier settings.

Calculation of Results The concentrations of the oxidised nicotinamide nucleotides in each whole brain extract were calculated by subtracting the relative fluorescence value for the tissue blank (F) from the relative fluorescence values for D and E, which represented respectively the NAD and NADP in the original extract (A) (Fig. 32). The total concentrations of these nucleotides in the whole brain extract were then determined by multiplying the corrected relative fluorescence values (D-F, E-F) by a calibration factor, which took into account the various dilutions and sampling operations made and the relationship between the nicotinamide nucleotide content and relative fluorescence, as indicated by the internal NAD standard (G). The calibration factor was therefore the product of two components.

The first of these was the ratio

$$\frac{\text{NAD Content of G (}\mu\text{M)}}{\text{Corrected Relative Fluorescence Value for G}} \quad \ldots\ldots\ldots \quad (1)$$

which converted the corrected relative fluorescence values for D and E into micromoles of NAD and NADP respectively. The concentrations of these nucleotides in the whole brain extract (A) were then calculated by multiplying the values obtained for the individual samples by the second ratio

$$\frac{10 \times 2.318}{0.5} \quad \ldots\ldots\ldots (2)$$

which was based upon the fact that a 2.0 ml aliquot (B/C), taken from the original (20 ml) whole brain extract (A) was diluted with reactants to 2.318 ml, from which 0.5 ml aliquots were then removed for assay.

A similar method was used to calculate the reduced nicotinamide nucleotide levels. In this case the calibration factor was the product of the following ratios

$$\frac{\text{NADP Content of M } (\mu M)}{\text{Corrected Relative Fluorescence Value for M}} \quad \ldots\ldots\ldots (1)$$

and

$$\frac{10 \times 2.332}{0.5} \quad \ldots\ldots\ldots\ldots\ldots (2)$$

Recovery Experiments These studies were carried out to measure the loss of oxidised and reduced nicotinamide

nucleotides occurring throughout the assay.

A brain was excised, divided into two equal and anatomically similar parts by cutting it longitudinally along the line of the median fissure, between the cerebral hemispheres. Each half was weighed and transferred to a labelled (either "test" or "control") Potter-Elvehjem glass homogeniser containing hot (90°C) TRIS buffer. At the same time a volume of TRIS buffer (0.5 ml), containing 0.074 µM of NAD was added to the homogeniser labelled "test". An equivalent volume of TRIS was added to the control homogeniser. The separate brain extracts (20 ml) were then prepared in the usual manner.

Aliquots (2 ml) of these extracts were treated in turn with acid and alkali to produce two samples equivalent to B/C (Fig. 32), which were then treated with glucose-6-phosphate and its dehydrogenase. Two aliquots (0.5 ml) from the test sample (B/C) and two from the control (B/C) were treated with acid to destroy the $NADPH_2$ (Fig. 32). In each case, one of the acid-treated samples, treated with alkali to destroy the remaining NAD, was used as a blank. All four samples

were incubated with the alkali-peroxide reagent, diluted with water and examined spectrophotofluorometrically.

The fluorescence produced by tissue NAD in the original control extract was calculated using the tissue blank. This value, expressed as relative fluorescence/g of brain tissue, was used to calculate the fluorescence produced by the NAD added to the test extract.

A further 0.5 ml sample from the control aliquot (B/C) was treated with acid and was mixed with a solution of NAD (0.0016 μM) in TRIS buffer (0.1 ml). This modified sample was incubated with the alkali-peroxide reagent, diluted with water and examined spectrophotofluorometrically. The fluorescence in this control sample was corrected by subtracting the tissue blank and was used to calculate the percentage recovery for NAD.

A similar procedure was used to determine the percentage recovery for $NADPH_2$. In this case the treatment used was similar to that described for H/I in Fig. 32.
Recoveries of 78%, 83% and 85% for NAD, and 69%, 72% and 83% for $NADPH_2$ were obtained.

Statistical Designs Used in the Assay of Nicotinamide Nucleotides Biological investigations may be affected by variations in technique and variability amongst animals. Although the overall effects of these factors can be considerably reduced by standardising procedures and selecting test animals of similar age, sex, strain and weight, they cannot be completely eliminated. The likelihood of one particular source of variation biasing the results may however be avoided by randomising the selection and treatment of test animals, and all subsequent operations. Statistical methods are also essential to estimate the experimental error and calculate the significance of differences between means.

In this investigation two experimental designs, relying upon different methods of randomisation, were used. In series 1 and 2, groups of three animals were arranged in three 3 x 3 latin squares (Appendix II). Each group contained one control rat, which was treated with saline and two others, which received different doses of the drug. In other experiments, using groups of two animals, randomisation was achieved by means of

a table of random numbers (Appendix II). Data obtained using these designs were evaluated by the appropriate variance analysis.

Computer Programming An ALGOL programme was devised to permit the time consuming and repetitive variance analysis calculations to be carried out using an English Electric KDF 9 computer. This programme calculated the means, the standard errors of the means and carried out a complete analysis of variance. Data, consisting of relative fluorescence values for drug, control, drug blank, control blank, together with the brain weights and the calibration factors, were transferred to 8-channel paper tape and used in this form in the computer (Appendix III).

Studies on the Incorporation of Radioactive Nicotinamide into Rat Brain and Liver NAD

Introduction The incorporation of nicotinamide into rat brain and liver NAD was measured by the modified method of Bonasera, Mangione and Bonavita (1965). Radioactive (carbonyl - ^{14}C, 13.2 mc/mM) and unlabelled nicotinamide were administered intraperitoneally together with either drug or control solution. After 3 hr, the rats were killed and trichloracetic brain and liver extracts were prepared and chromatographed on an ion-exchange resin (Appendix V) to separate the NAD, which was then assayed spectrophotofluorometrically. Radioactivity levels in the NAD fractions were also measured and the rate of incorporation of nicotinamide (carbonyl - ^{14}C) into NAD was calculated by determining the counts / μM of NAD / g of tissue.

Preliminary Investigations Certain experiments were carried out to determine the effectiveness of this method of ion-exchange chromatography in isolating NAD.

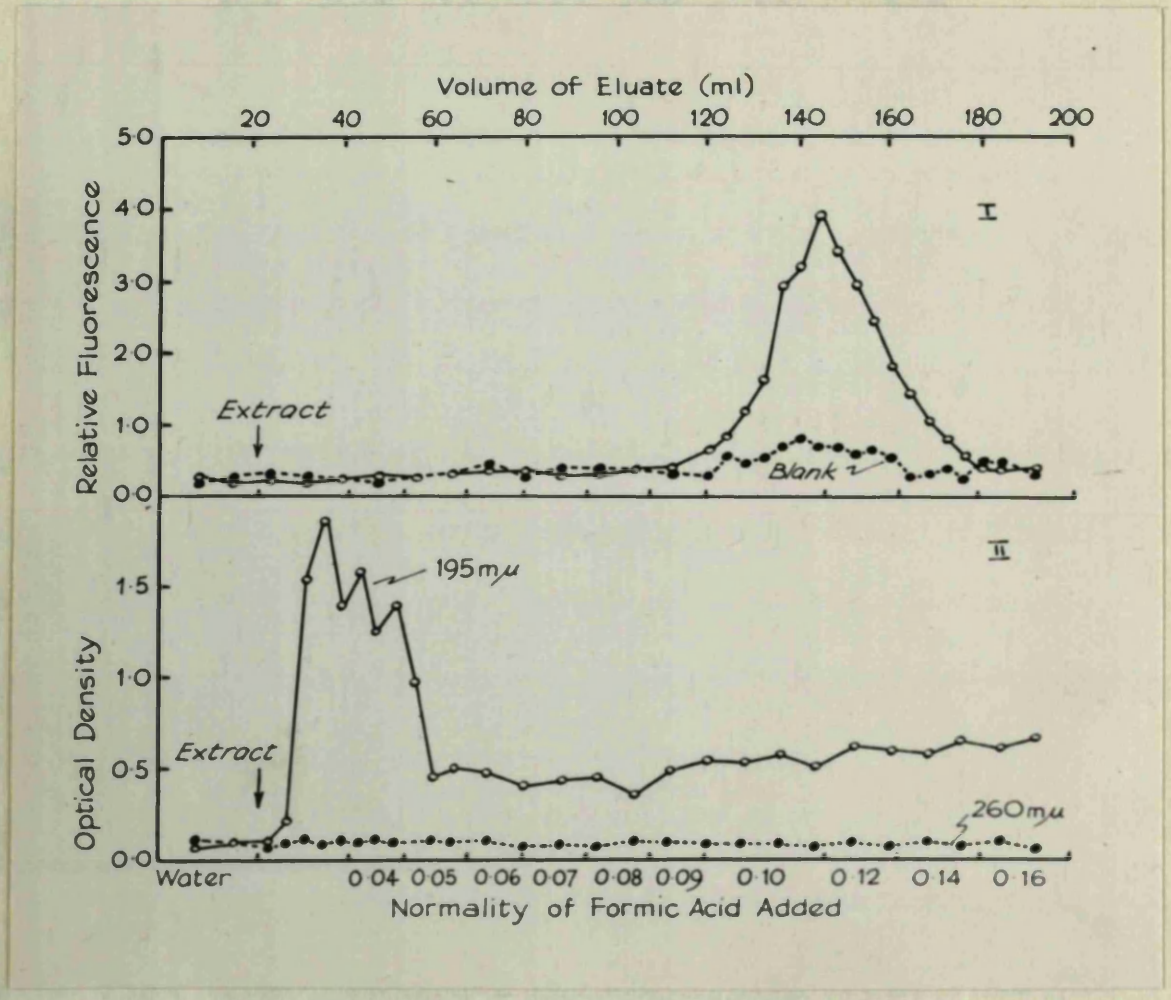

Fig. 34. Relative Fluorescence and Optical Density Readings Obtained Using Brain Eluate Samples.

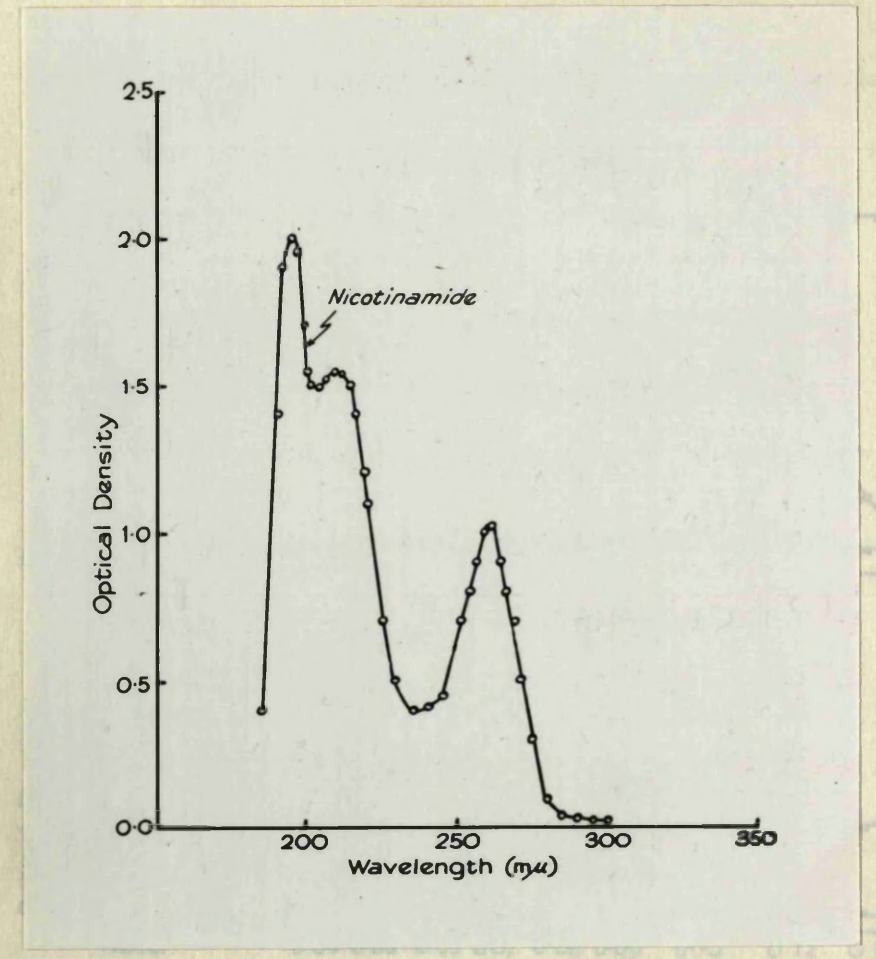

Fig. 35. U.V. Spectrum of Nicotinamide.

In the first of these studies a rat brain was excised, weighed, transferred to ice-cold trichloracetic acid (15 ml, 5% w/v), homogenised and centrifuged (20,000 x g, 30 min, 0°C). The pH of the separated supernatant was titrated to 7.5 using sodium hydroxide (0.5 N, 0°C). The final volume was then adjusted to 20ml.

This extract was chromatographed at a constant rate (0.25 ml/min) (Appendix V). A stepwise elution was carried out using successive volumes of formic acid, increasing in concentration from 0.04 N to 0.16 N(Fig. 34). The eluate was collected continuously in 2 ml fractions. Aliquots (0.2 ml) from these samples were then added to water (2 ml) in a spectrophotometer cell (1 cm) and optical density measurements made at two different wavelengths, reading against a water blank. This procedure was adopted since it permitted the identification of nicotinamide, which has absorption maxima at 195 mμ and 260 mμ (Fig. 35). Initial observations showed that the normal brain extract eluate contained insufficient nicotinamide to be detected by this technique, since no parallel peaks were apparent in the 195 mμ and 260 mμ

graphs (Fig. 34, II). This experiment also showed that certain unidentified substances with absorption maxima at 195 mμ either passed directly through or were eluted from the column by 0.04 N formic acid (Fig. 34, II).

Two 0.3 ml aliquots were removed from each fraction of eluate and transferred to glass centrifuge tubes (10 ml) in preparation for the spectrophoto-fluorometric estimation of NAD. Blanks were produced by treating one aliquot from each fraction with 0.5 ml of sodium hydroxide (N) in a water bath (60°C, 15 min). All the samples were then incubated with 1.5 ml of the combined sodium hydroxide (8N) - hydrogen peroxide (0.02%) reagent at 38°C for 1 hr. After diluting with water to 8.0 ml, the fluorescence of each sample was measured at activating and fluorescent wavelengths of 360 mμ and 465 mμ respectively. This experiment confirmed that NAD was eluted from the column by 0.1 N formic acid (Fig. 34, I). However, further experiments were considered necessary to determine whether these two concentrations (0.04 N, 0.10 N) of formic acid alone were sufficient to produce a satisfactory separation between

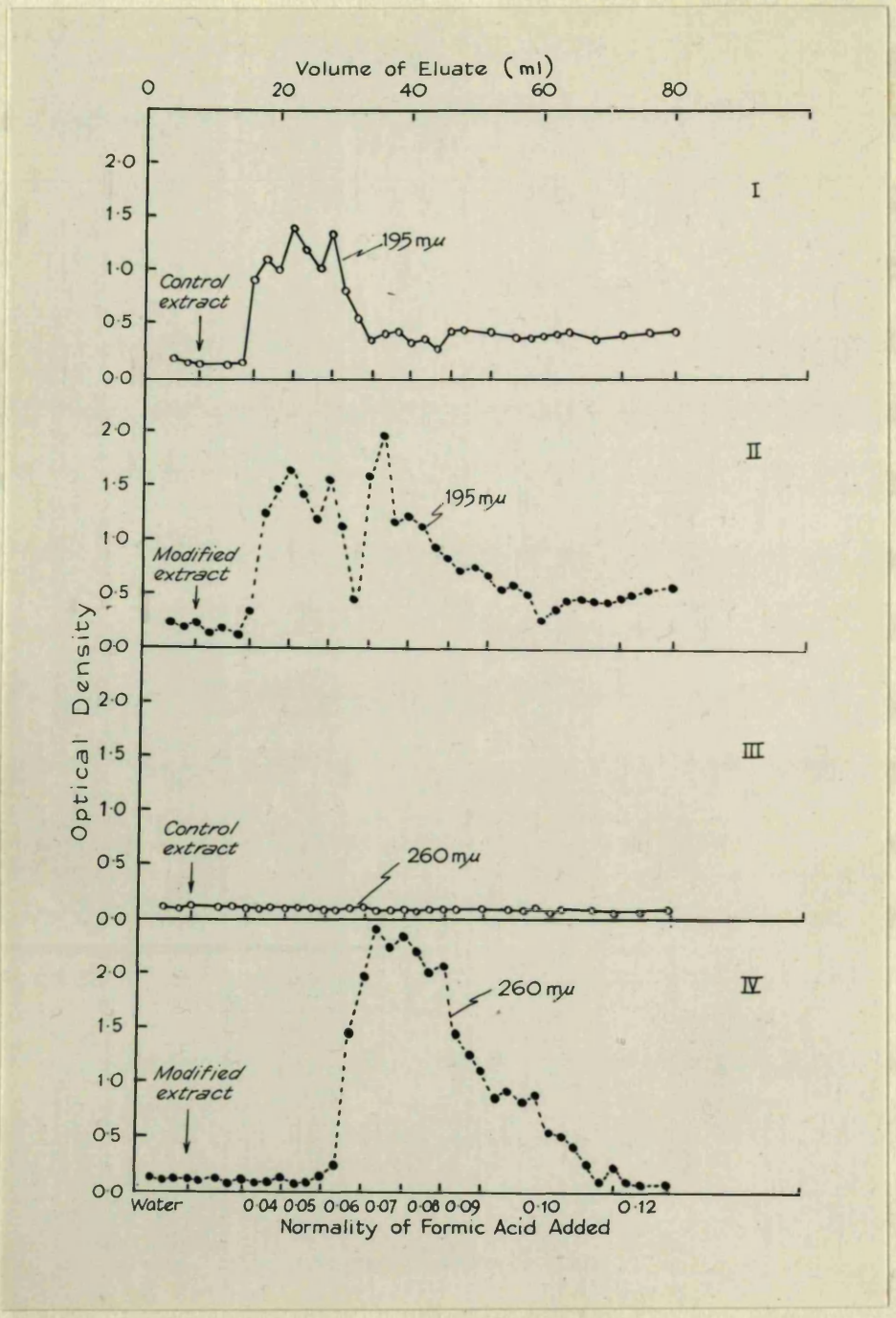

Fig. 36. Optical Density Readings Obtained Using Eluate Samples from Control and Nicotinamide-Treated Brain Extracts.

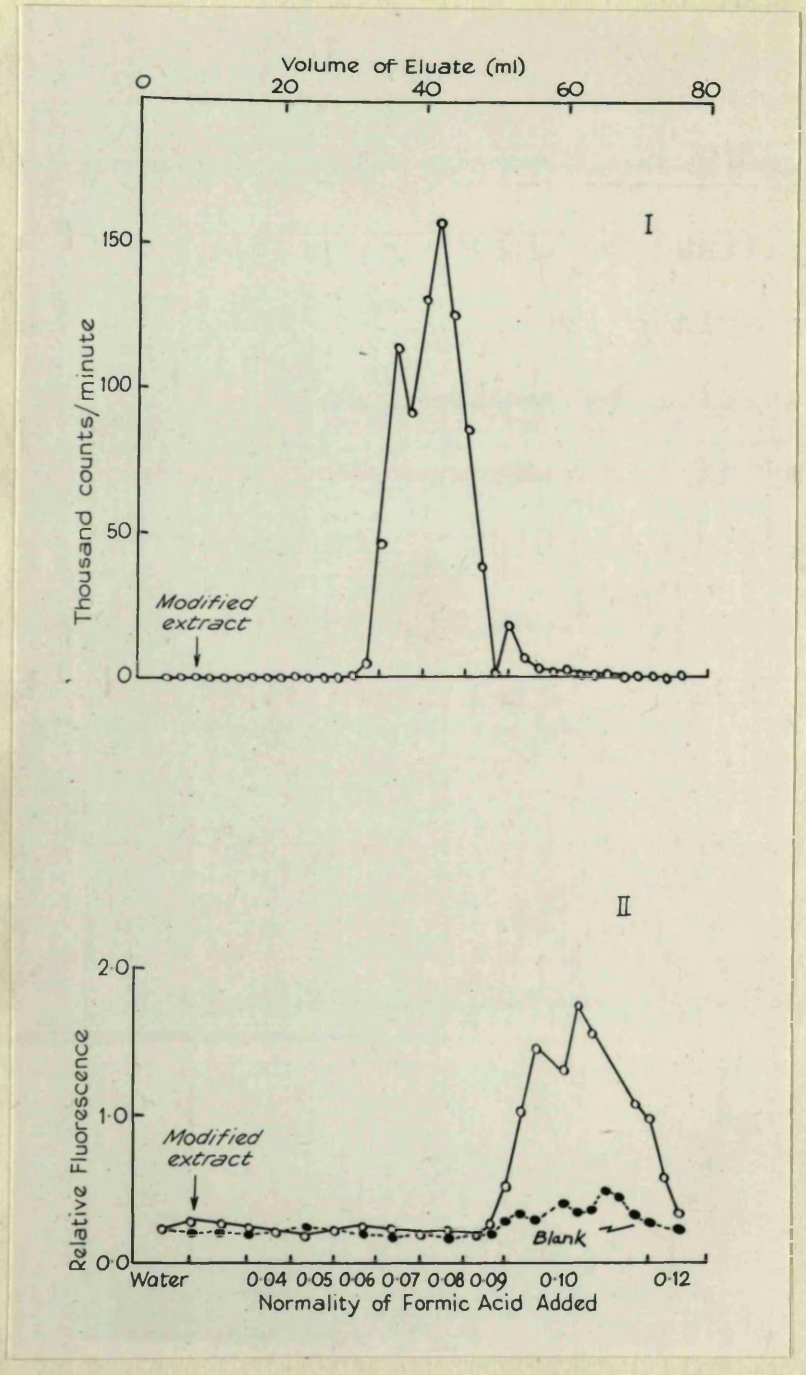

Fig. 37. Radioactivity and Relative Fluorescence Values for Samples of Eluate from the Nicotinamide-Treated Brain Extract.

NAD and the nicotinamide likely to be present in brain extracts of nicotinamide-treated rats.

A neutralised trichloracetic acid extract of brain was divided into two equal volumes (10 ml). One of these was modified by adding 2.0 ml of a solution containing 50 mg of nicotinamide and 0.01 mc of radioactive nicotinamide (13.2 mc/mM). A control was prepared by adding 2.0 ml of water to the second aliquot. These extracts were chromatographed separately on Dowex-1-formate columns and eluted using increasing concentrations of formic acid (0.04 N to 0.12 N). Fractions of eluate were collected at regular intervals and measurements made of the optical densities (Fig. 36), the induced fluorescence (Fig. 37) and the radioactivity (Fig. 37) of each sample.

Elution of the control extract gave optical density readings similar to those obtained previously (Fig. 34). A peak was observed in the 195 mμ graph (Fig. 36, I) with no corresponding increase in the 260 mμ graph (Fig. 36, III), which remained unaltered throughout the elution. On the other hand elution of the modified extract showed a prolonged irregular rise in optical

Fig. 38. Infra-Red Lamps Used to Dry Eluate Samples.

density at both 195 mμ (Fig. 36, II) and 260 mμ (Fig. 36, IV), in addition to the normal rise at 195 mμ (Fig. 34). The parallel increases in optical density first appeared during elution with 0.06 N and continued until elution with 0.09 N formic acid had been completed (Fig. 36, II and IV).

Aliquots (1 ml) from each sample of eluate were diluted to 8.0 ml with water, transferred to aluminium planchets and dried using radiant heat supplied by 6 infra-red reflector lamps (Osram, 200v - 250v, 250w) (Fig. 38). The radioactivity in each planchet was counted for 3 min using a proportional gas flow counter (Tracerlab). These results (Fig. 37, I) confirmed that nicotinamide was largely eluted by concentrations of formic acid between 0.06 N and 0.09 N since the radioactivity peak obtained within this range corresponded to the parallel rises in optical density at 195 mμ and 260 mμ (Fig. 36, II and IV).

The fluorescence measurements (Fig. 37, II) indicated that NAD was eluted by concentrations (0.09 N to 0.12 N) just greater than those required to elute nicotinamide (0.06 N to 0.08 N).

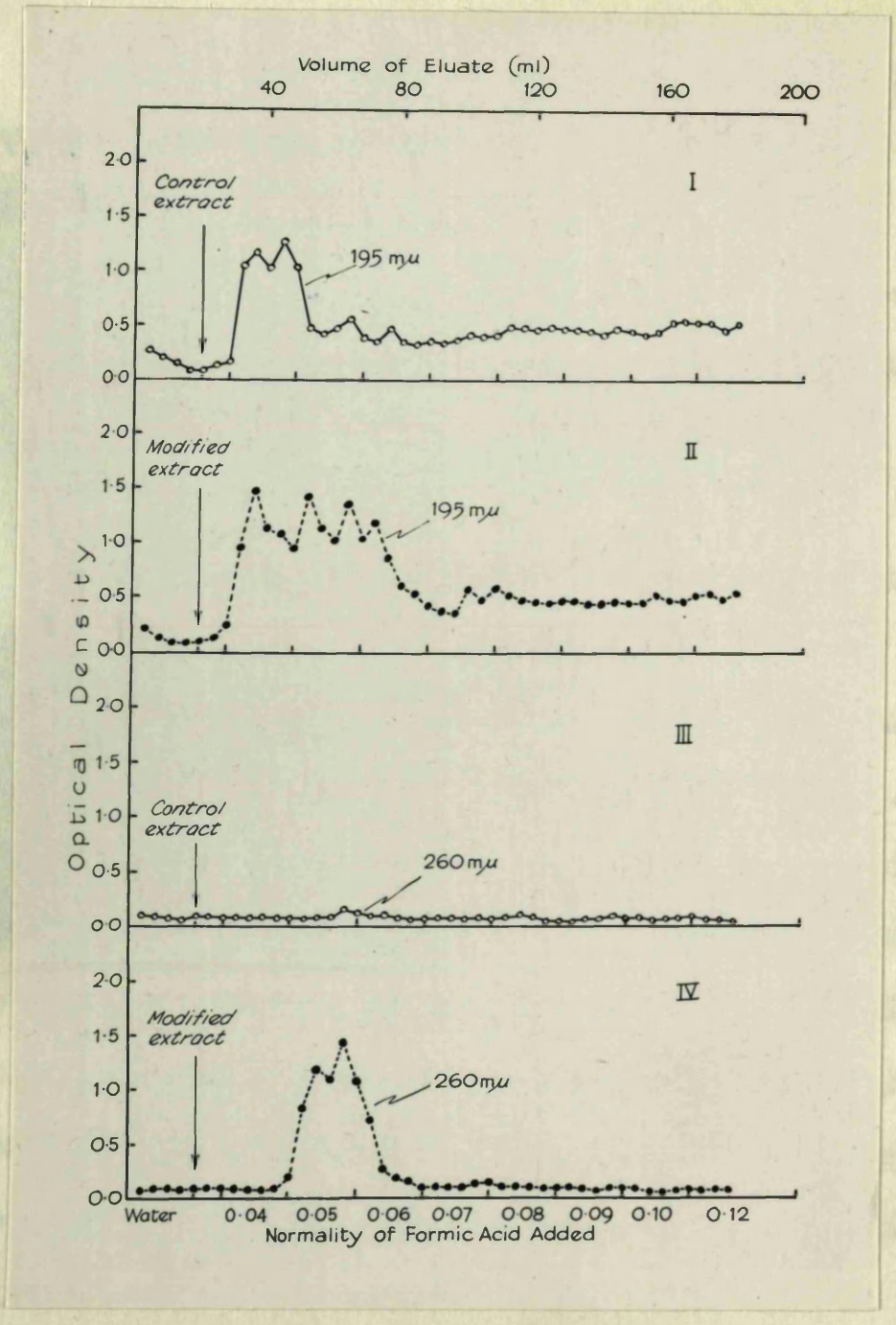

Fig. 39. Optical Density Readings Obtained Using Eluate Samples from Control and Nicotinamide-Treated Brain Extracts.

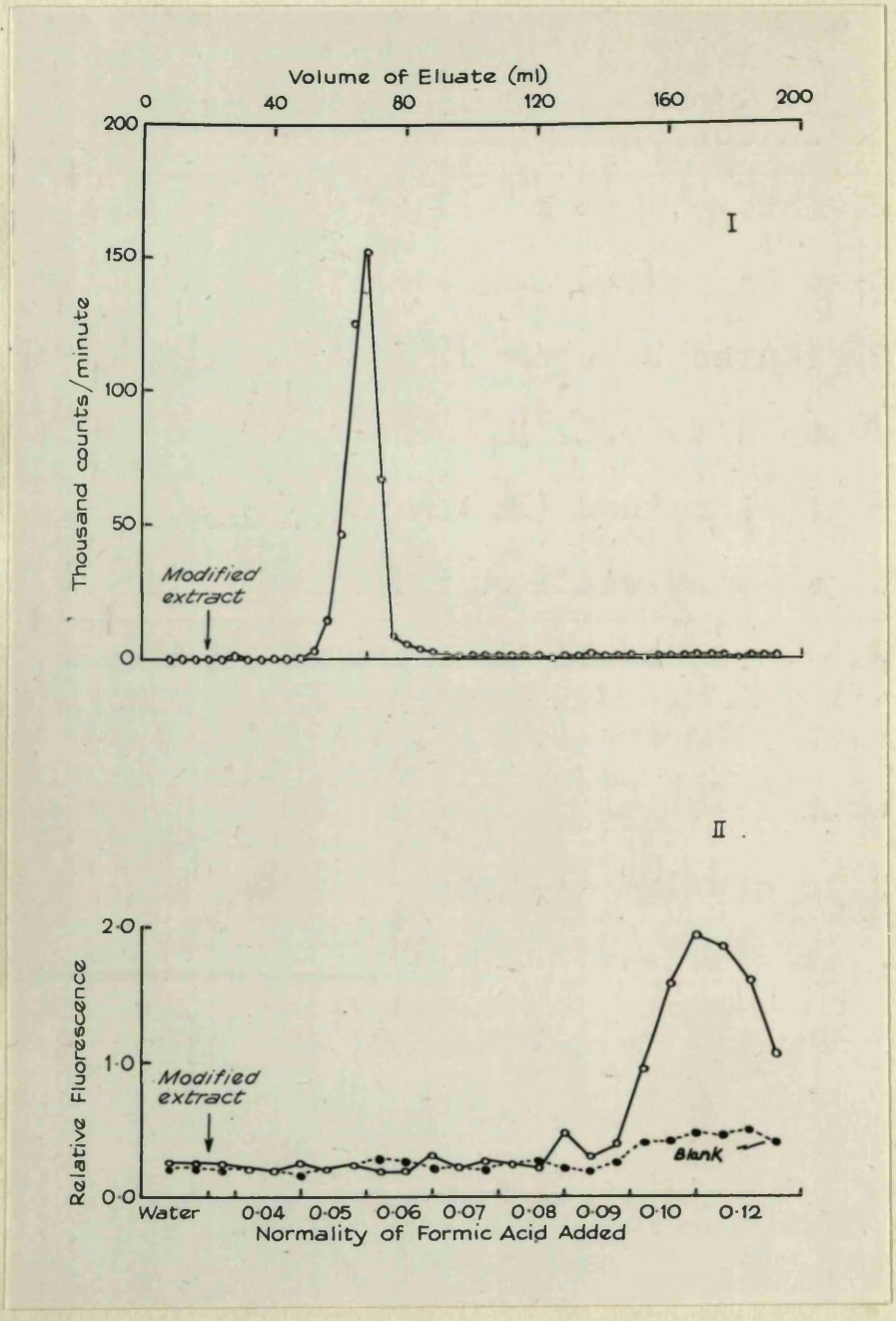

Fig. 40. Radioactivity and Relative Fluorescence Values for Samples of Eluate from the Nicotinamide-Treated Brain Extract.

Another similar experiment using larger volumes of each concentration of formic acid, produced a more satisfactory separation of nicotinamide (Fig. 39, II and IV) and NAD (Fig. 40, I and II), which were respectively eluted by concentrations of 0.05 N to 0.06 N and 0.09 N to 0.12 N. These results showed that the original method (Bonasera *et al*, 1965), which contained a two-step elution, using only 0.04 N and 0.10 N formic acid, would be improved by incorporating an intermediate elution with 0.06 N formic acid.

A number of *in vivo* experiments were then carried out to confirm that the modified technique produced a satisfactory separation of NAD and that this nucleotide could be suitably labelled using radioactive nicotinamide (carbonyl ^{14}C). A rat was injected with 2 μc of radioactive nicotinamide, mixed with unlabelled nicotinamide (250 mg/kg). After 3 hr the brain and part of the liver (1.0 g) were excised and trichloracetic acid extracts of them were prepared. The liver supernatant was mixed with 5 volumes of cold acetone (50 ml, -20°C) to precipitate the NAD. After 12 hr (-20°C) the precipitate produced by this treatment was

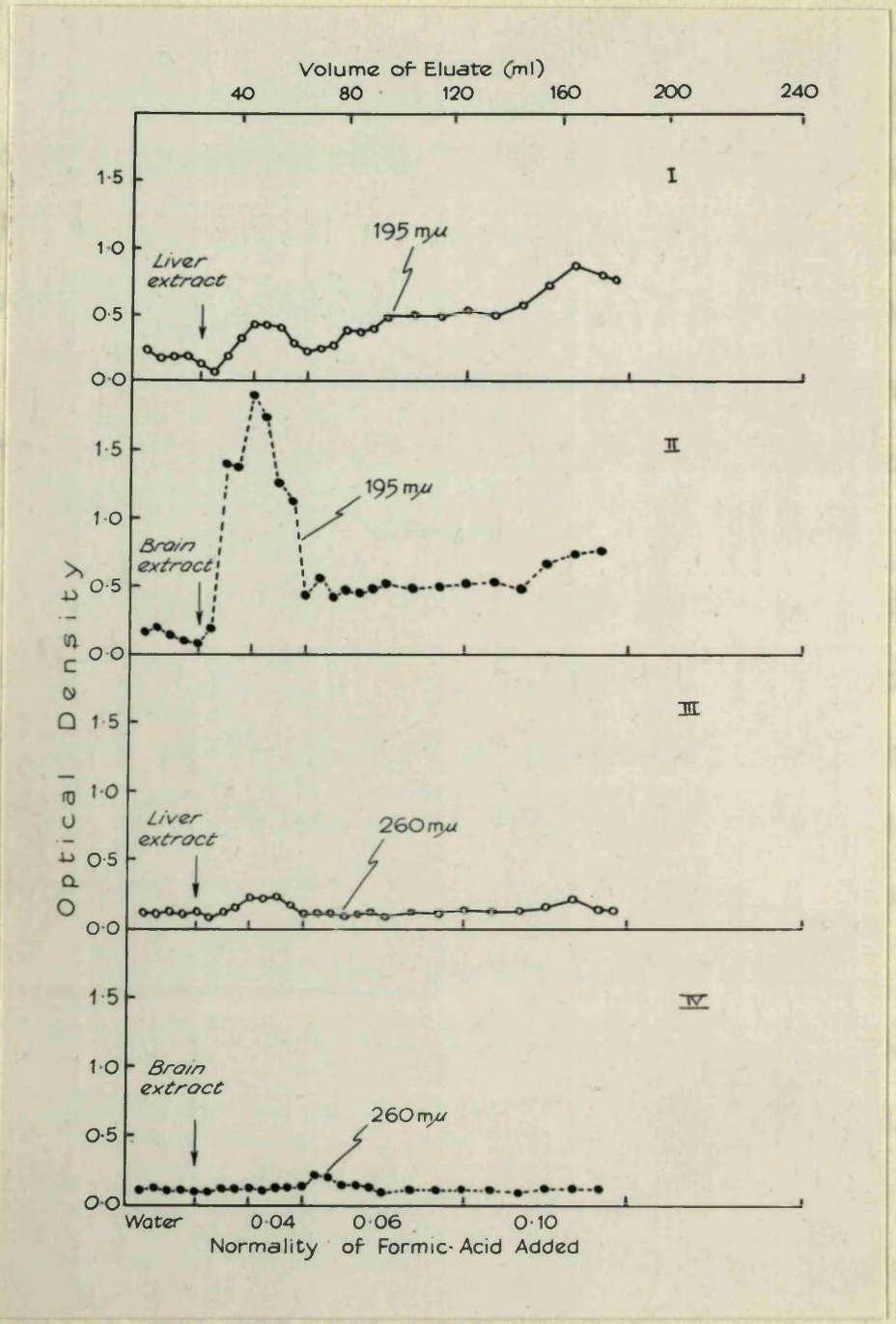

Fig. 41. Optical Density Readings Obtained Using Eluate Samples from Brain and Liver Extracts of a Nicotinamide-Treated Rat.

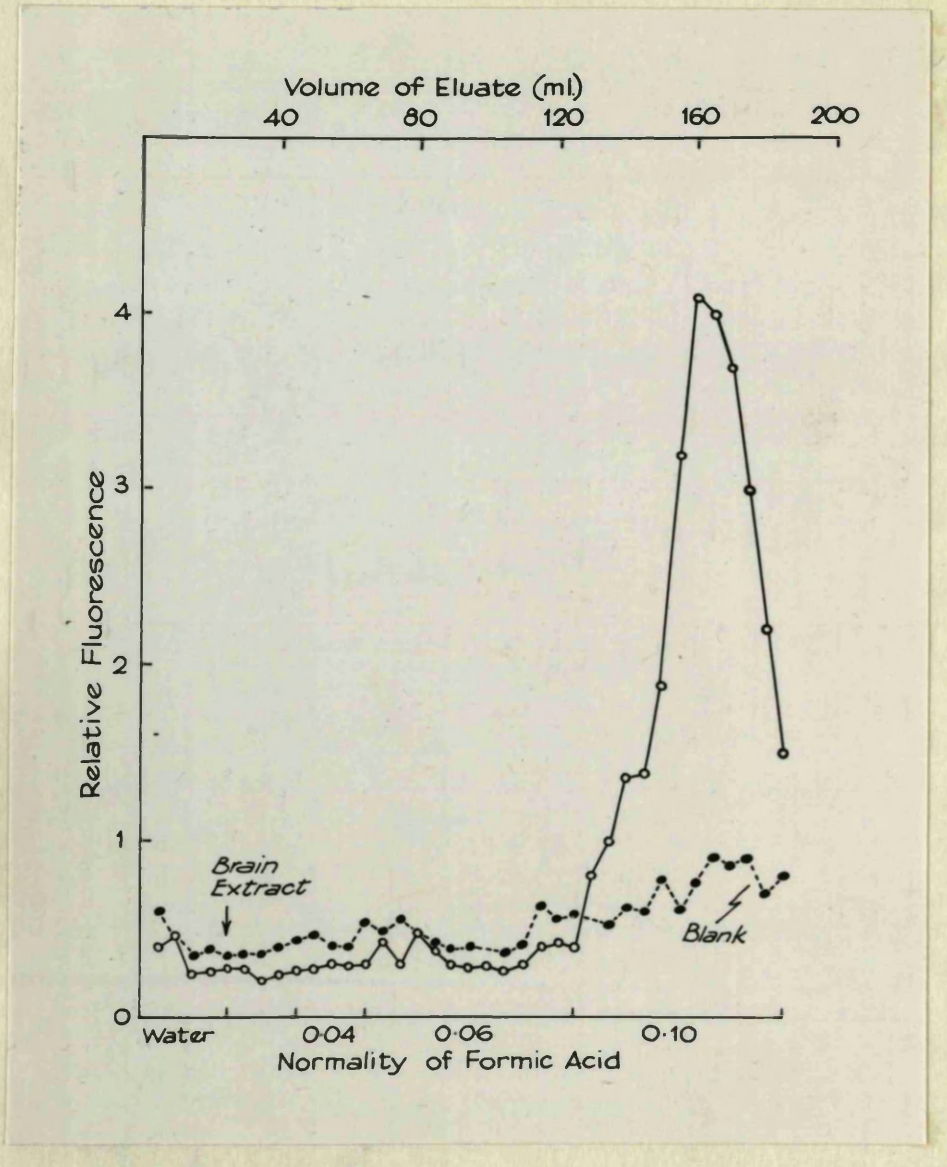

Fig. 42. Fluorescence Values in Eluate Samples from the Brain Extract of a Nicotinamide-Treated Rat.

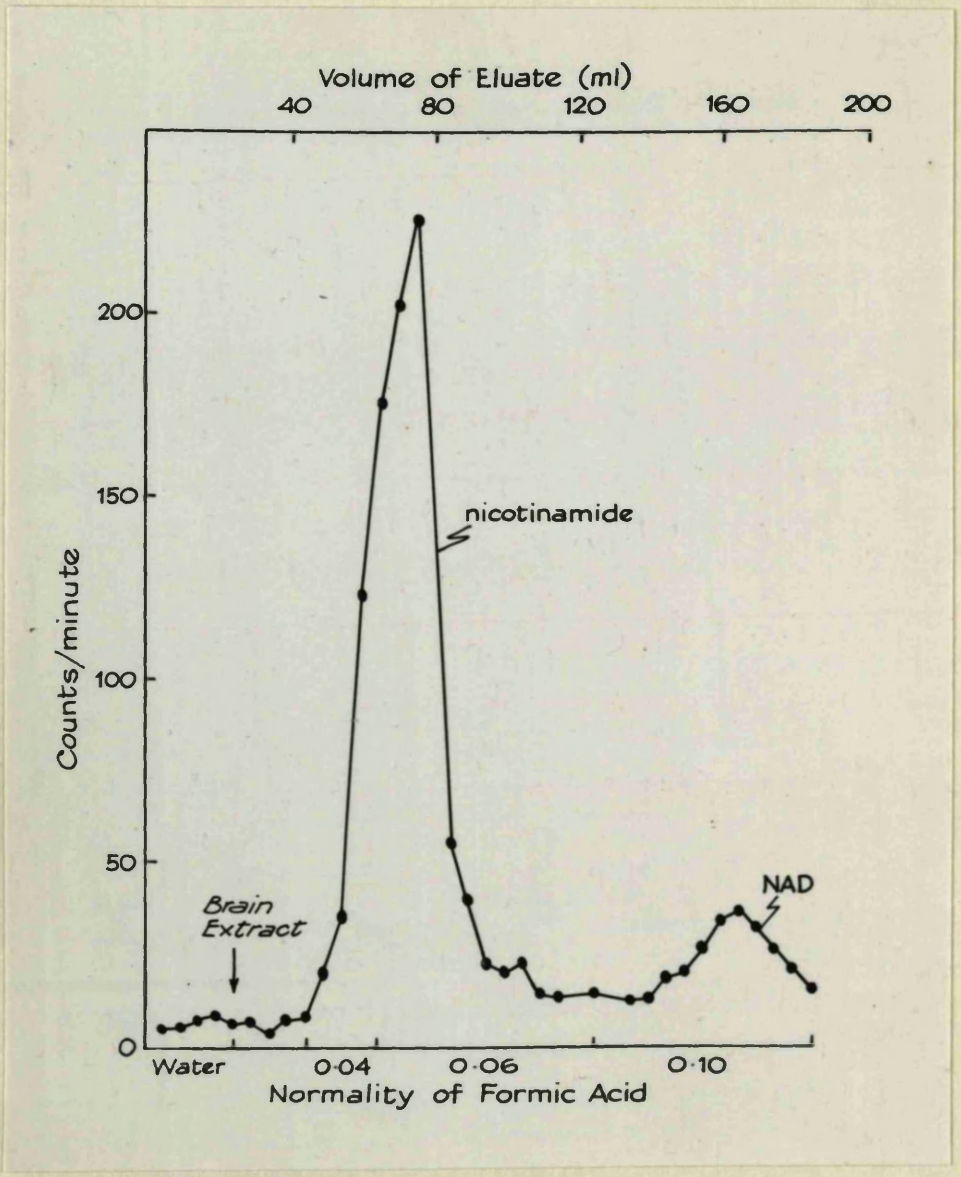

Fig. 43. Radioactivity Levels in Eluate Samples from the Brain Extract of a Nicotinamide-Treated Rat.

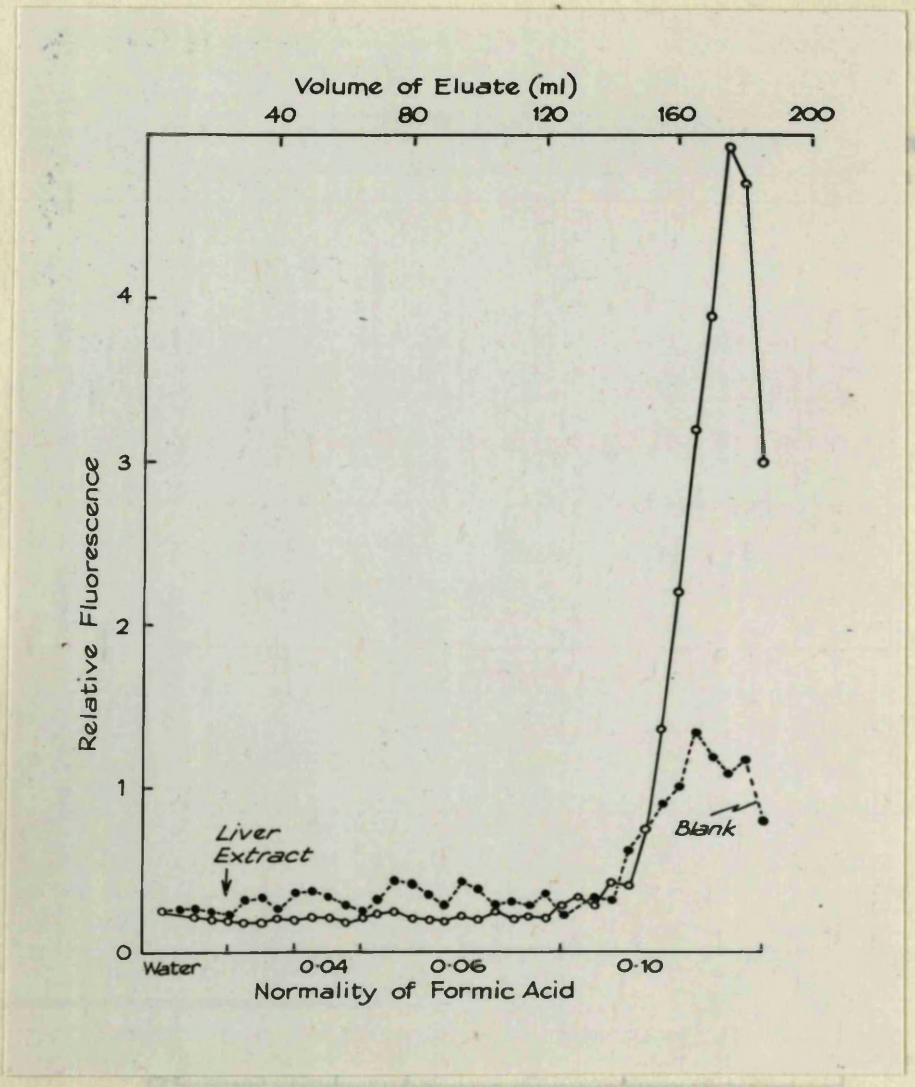

Fig. 44. Fluorescence Values in Eluate Samples from the Liver Extract of a Nicotinamide-Treated Rat.

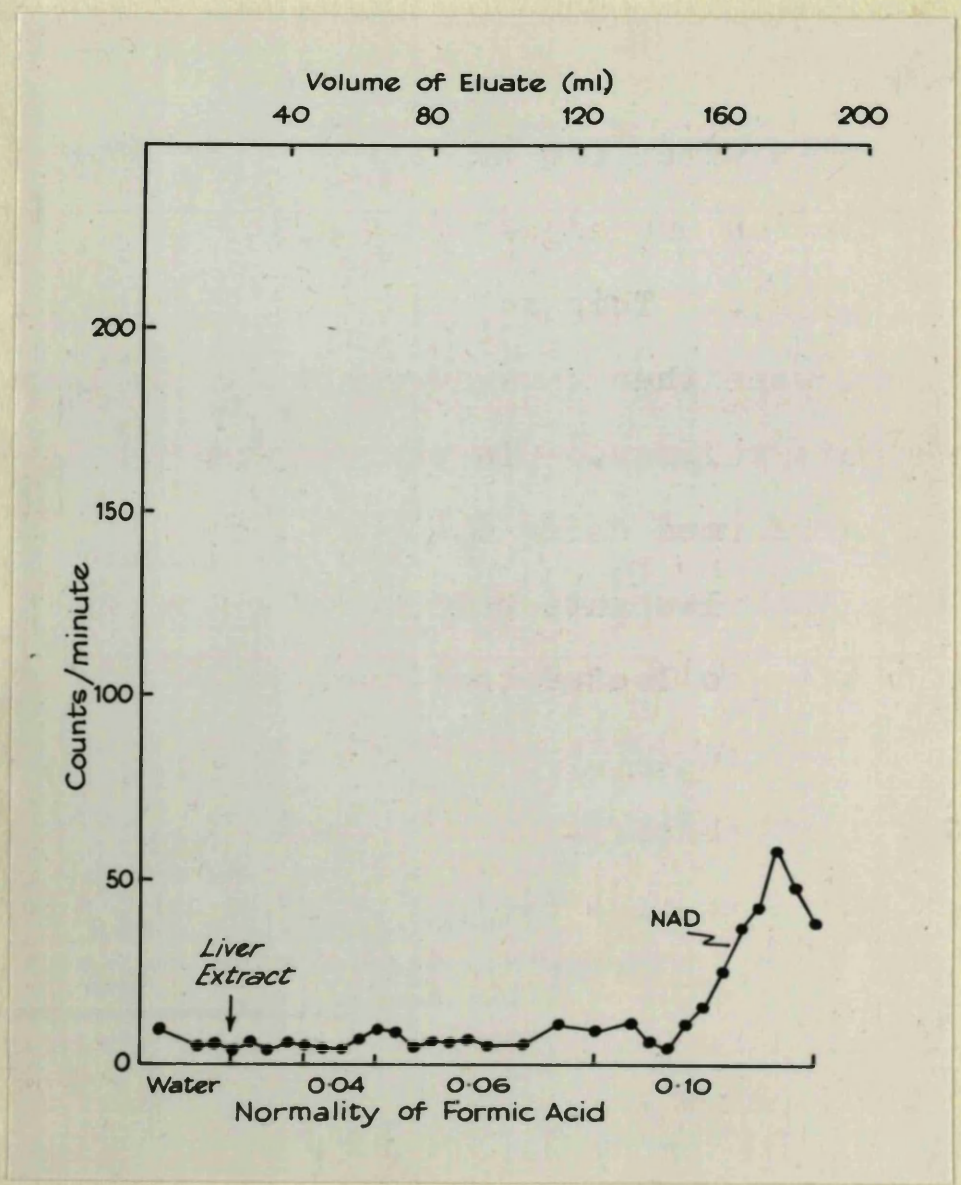

Fig. 45. Radioactivity Levels in Eluate Samples from the Liver Extract of a Nicotinamide-Treated Rat.

removed by centrifuging (25,000 xg, 30 min, -20°C) and was dissolved in water (20 ml, 2°C). The pH of the resulting solution was adjusted to 7.5 with sodium hydroxide (0.1 N). This solution and the neutralised brain extract were then chromatographed on separate Dowex-1-formate columns. In each case a 3-stage elution was performed using 0.04 N, 0.06 N and 0.10 N formic acid, the flow rate being adjusted to 0.25 ml/min. The eluates were collected continuously in 5.0 ml fractions, which were examined spectrophotometrically (Fig. 41, I to IV), spectrophotofluorometrically (Fig. 42, Fig. 44) and scanned to determine the radioactivities (Fig. 43, Fig. 45).

This experiment demonstrated that quantities of brain nicotinamide, too small to be detected satisfactorily using optical density measurements (Fig. 41, II and IV), could be separated from NAD by a 3-stage formic acid elution (Fig. 42, Fig. 43). Examination of the liver eluate (Fig. 45) showed that nicotinamide was completely removed by cold acetone precipitation. In both the brain and liver eluates small peaks of radioactivity (Fig. 43, Fig. 45), corresponding exactly to the NAD

fluorescence peaks (Fig. 42, Fig. 44), showed that a certain proportion of the administered radioactive nicotinamide had been incorporated into NAD.

Recovery Experiments The recovery of NAD from the Dowex-1-formate column was measured using a brain extract. This extract was divided into two equal volumes (10 ml), one of which was modified by adding NAD (0.1 μM). A control was prepared by adding an equal volume of water to the second aliquot. These extracts were then chromatographed at a constant rate (0.25 ml/min) on separate Dowex-1-formate columns, each of which was eluted in turn with 50 ml of 0.04 N, 50 ml of 0.06 N and 100 ml of 0.10 N formic acid. NAD (0.1 μM) was then added to the 0.10 N fraction of the control eluate.

Two aliquots (0.5 ml) were removed from each 0.10 N fraction. In each case one aliquot was incubated with 0.5 ml of sodium hydroxide (N) for 15 min at 60°C to produce a blank. These blanks together with the untreated aliquots were incubated with 1.5 ml of the sodium hydroxide (8 N) - hydrogen peroxide (0.02%) reagent for 1 hr at 38°C. After diluting each

sample to 8.0 ml with water, 3.0 ml aliquots were transferred to cells (1 cm) and examined in a spectrophotofluorometer. Having corrected for the blanks, the percentage recoveries were calculated. Recoveries of 100.4%, 100.9% and 100.7% showed that this method of isolating NAD was satisfactory. No correction was made for this slight discrepancy in subsequent experiments.

Measurement of Nicotinamide (^{14}C) Incorporation into NAD

Groups of rats were injected intraperitoneally with radioactive nicotinamide (100 μc), mixed with the unlabelled nicotinamide (250 mg/kg). After 10 min the animals were treated with either drug or saline solution by the same route. The rats were killed 3 hr later. Trichloracetic acid extracts of the whole brain and part of the liver (approximately 1.5 g) from each rat were prepared and chromatographed on Dowex-1-formate at a constant flow rate (0.25 ml/min). A 3-stage elution was then performed with formic acid (50 ml of 0.04 N, 50 ml of 0.06 N and 100 ml of 0.10 N), NAD being recovered in the third stage.

Three 0.5 ml aliquots from each 0.10 N formic acid fraction of eluate were transferred to labelled test tubes, embedded in an ice-bath. A standard was prepared for the fluorometric assay by adding 0.5 ml of NAD solution (0.01 μM) to one aliquot. A blank was produced by incubating a second aliquot with 0.5 ml of sodium hydroxide (N) for 15 min at 60°C. The third aliquot was mixed with 0.5 ml of water. These samples were then treated with the sodium hydroxide (8N) - hydrogen peroxide (0.02%) reagent in the usual manner. After diluting each sample to 8.0 ml with water, a 3.0 ml aliquot was transferred to a cell (1 cm) and examined spectrophotofluorometrically at activating and fluorescent wavelengths of 360 mμ and 465 mμ respectively. In each case the relative fluorescence value, representing the product of the percentage transmission and the meter multiplier setting, was corrected by subtracting the blank and was converted into micromoles, using the internal NAD standard.

An 8.0 ml aliquot from each 0.1 N fraction of eluate was transferred to a planchet and dried using

infra-red lamps (Fig. 38). The radioactivity was counted in a proportional gas flow counter. The final results were corrected for the background radioactivity by subtracting the count obtained for the empty planchet at the beginning of the experiment. The results were then expressed as counts/μM of NAD/g of tissue.

The results were finally evaluated by means of a t-test, which was carried out using a digital (KDF 9) computer (Appendix III).

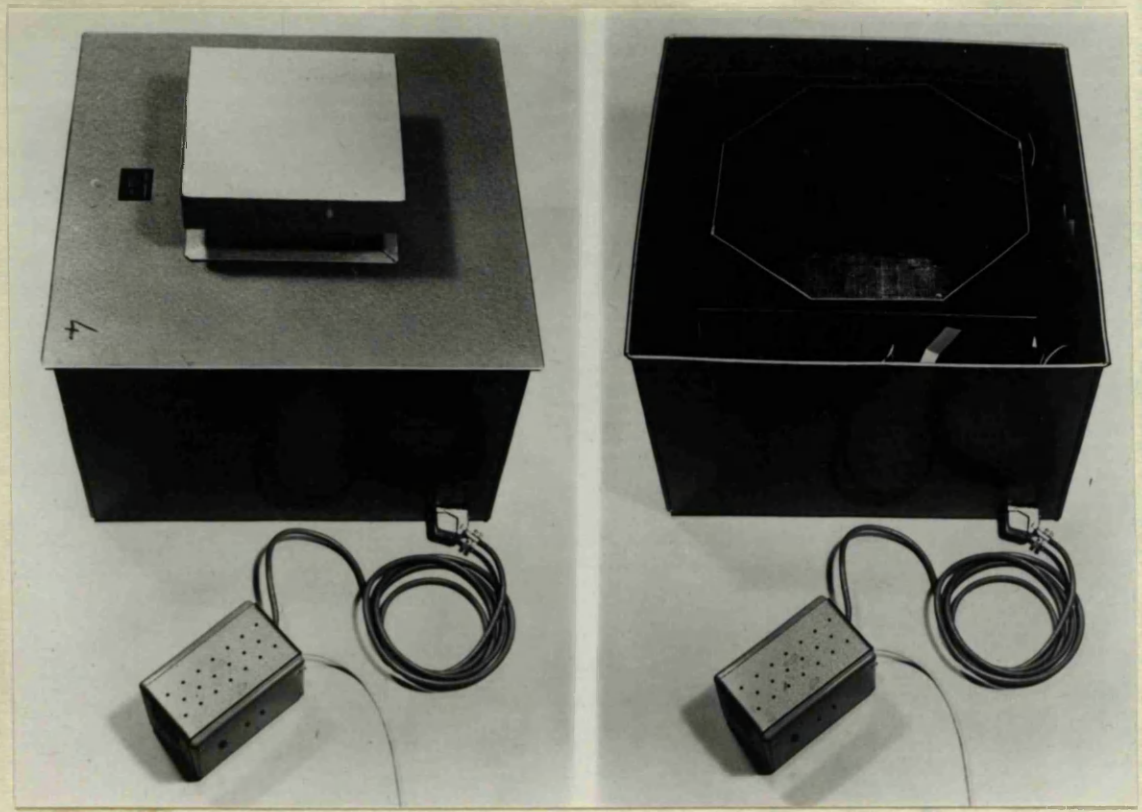

Fig. 46. Activity Box Showing the Lid With Ventilator, the Outer and Inner Walls, the Additional Octagonal Wall and the Counting Unit.

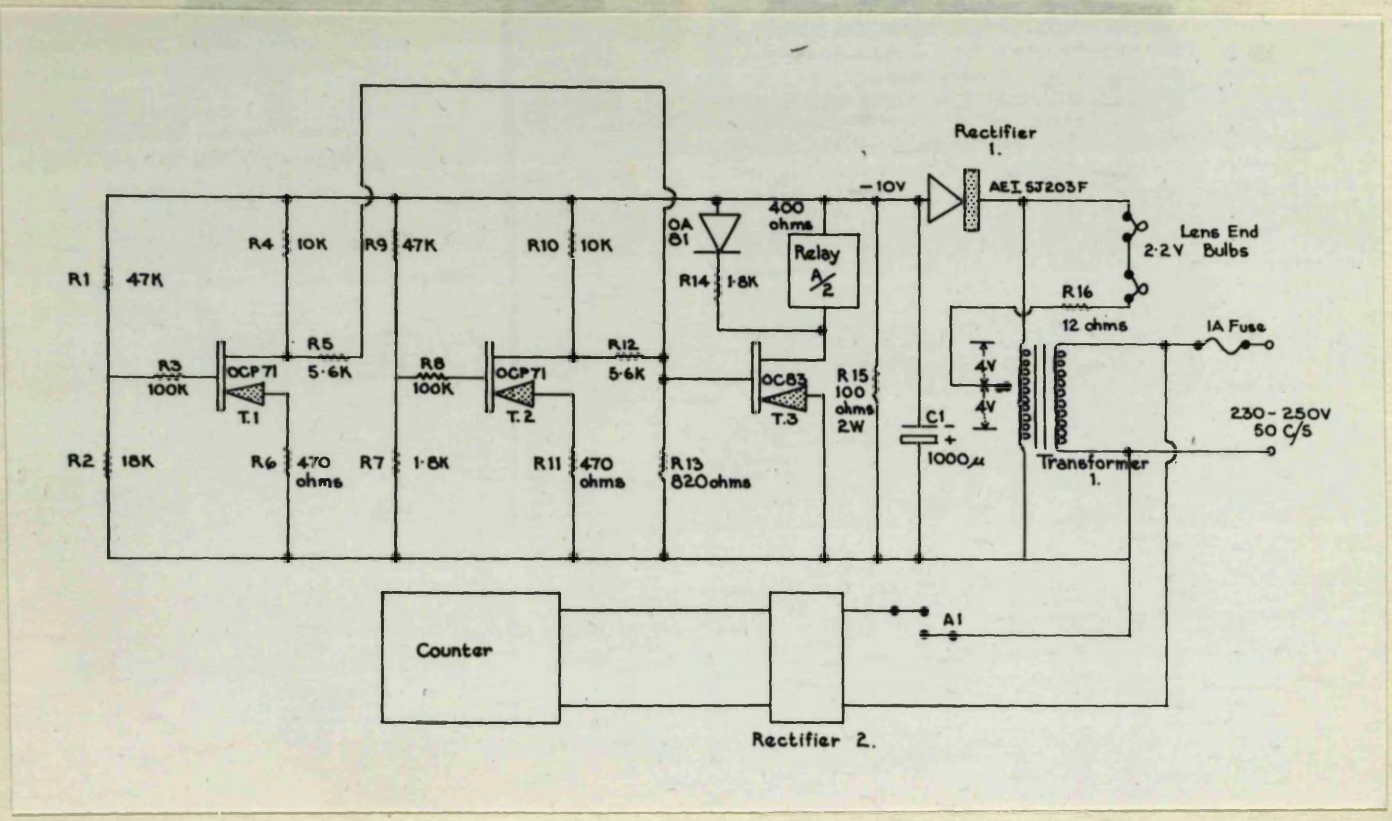

Fig. 47. Circuit Diagram of the Activity Box.

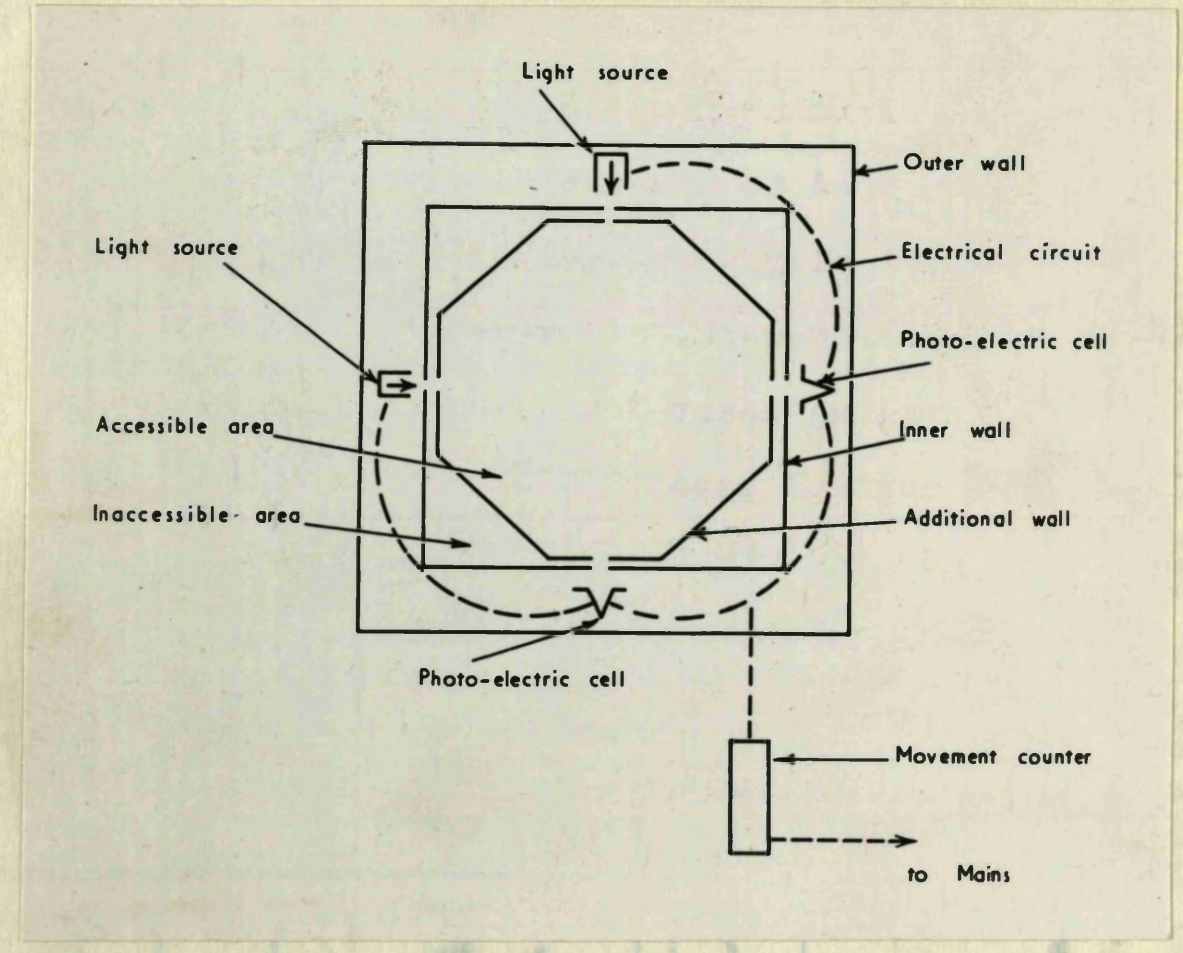

Fig. 48. Diagram of the Activity Box (Plan View).

Activity Studies

Apparatus Used Initially in this study, a simple "jiggle-cage" was used to measure locomotor activity. It consisted essentially of a small wire cage, suspended by springs from a framework. Movements were recorded by means of a Starling heart lever, arranged to write upon a smoked kymograph paper. This method was rejected at an early stage since the results were frequently exaggerated by the additional movements imparted by the freely-moving springs.

A fixed apparatus, consisting of a counting unit and a box containing two photo-electric cells was used instead. This activity box (Fig. 46), (35 cm x 35 cm in area, 30.5 cm high) was protected by an outer case containing the electrical circuit (Fig. 47). In each box two light beams crossed at right angles, just above the inner floor level. These impinged upon photo-electric cells situated equidistant from the corners and directly opposite the corresponding light sources (Fig. 48). The apparatus was covered by a lid (Fig. 46), which permitted adequate ventilation but did not allow

light to enter. In order to prevent the rats remaining in the corners, where they could move freely without crossing the light beams, an additional octagonal wall, with sides 14 cm long, was placed within the box, thereby rendering the corners inaccessible.

Method Used The experiments were carried out at 18°C in a quiet room using two activity boxes at a time. Male Wistar rats of equal body weight were selected for each investigation. Each animal was transferred to an activity box, where it remained for a preliminary control period of 1 hr before receiving either the drug or control solution. Animals which did not show a characteristic reduction in activity during this time were rejected.

In each complete experiment two groups of animals were treated intraperitoneally with either drug or control solution. Readings were taken every 15 min for 6 hr. The final results, calculated as the means for drug and control at each time interval, were expressed graphically. The significances of the differences between drug and control means at selected time intervals were determined using a t-test (Appendix III).

Mitochondrial Swelling Studies

Preparation of Mitochondria The method selected for this investigation was developed specifically for isolating brain mitochondria (Løvtrup & Zelander, 1962).

Rat brains were excised, rinsed in sucrose solution (0.44 M, 0°C) to remove excess blood and weighed. A 10% w/v homogenate was prepared in sucrose (0.44 M, 0°C), using a Potter-Elvehjem glass homogeniser, embedded in an ice-bath. After homogenising for 10 min, the suspension was centrifuged (2,100 xg, 10 min, 0°C). The supernatant was set aside at 0°C and the precipitate resuspended in sucrose (0.44 M, 0°C) and centrifuged (2,100 xg, 10 min, 0°C). The combined supernatants were then centrifuged (14,500 xg, 15 min, 0°C) and the precipitate resuspended in sucrose (0.44 M, 0°C) and centrifuged (7,000 xg, 15 min, 0°C). This washing operation was repeated twice. The precipitate obtained from the last centrifugation was resuspended in sucrose (0.25 M, 0°C) so that 1 ml of the final suspension represented 0.5 g of brain tissue (Kaufman & Kaplan, 1960).

Measurement of Mitochondrial Swelling Two methods of measuring mitochondrial swelling were used.

1. Spectrophotometric Method Aliquots (2 ml) of the suspension were added to a series of test tubes, each containing 0.5 ml of sucrose (0.25 M), potassium phosphate (0.1 M, pH 7.5) and glucose (0.02 M). These tubes were incubated at 30°C with constant shaking in a Warburg apparatus. At appropriate time intervals each tube was removed, chilled to 0°C and an aliquot (0.05 ml) transferred to 2.95 ml of EDTA (0.05 M) solution in a spectrophotometer cell (1 cm). Mitochondrial swelling was then measured by determining the optical density of the diluted aliquot spectrophotometrically at 520 mμ.

Drugs under investigation were dissolved in the incubation solution (sucrose, 0.25 M, potassium phosphate, 0.1 M, pH 7.5 and glucose, 0.02 M). A comparison between the optical density changes in the drug-treated and control suspensions was made at each time interval. Each experiment was carried out at least three times and the mean results for drug and

control were finally plotted against time.

2. Gravimetric Method Aliquots (5 ml) of the final mitochondrial suspension were transferred to weighed stainless steel centrifuge tubes, cooled to 0°C in an ice-bath. Equal volumes (1.5 ml) of either the incubating medium or a solution of the drug in it were added to the mitochondria, which were then incubated at 30°C in a Warburg apparatus.

At appropriate time intervals throughout the experiment, tubes were removed, cooled to 0°C and centrifuged (0°C). The centrifugal force was increased rapidly to 25,000 xg within 1.5 min and was maintained at this level for 4 min. Centrifugation was then stopped as quickly as possible (within 3.5 min) and the supernatants were discarded. The inner surfaces of the tubes were dried carefully using filter paper. Having replaced the caps, the centrifuge tubes were weighed, heated in an oven (100°C, 12 hr) and then reweighed when their contents were dry and cool. The water content of the mitochondria was finally calculated from these results.

Mitochondrial Staining

A small volume of the mitochondrial suspension was transferred from each tube to a glass microscope slide, where it was smeared evenly over the surface and allowed to dry at room temperature. Approximately 0.5 ml of a solution of Janus Green B in isotonic saline was pipetted on to the slide. After 10 min staining the excess dye was washed off using water. The slide was then dried at room temperature and was examined microscopically.

Photographing Mitochondria

Suitable fields were photographed using an Exacta Varex, 35 mm single lens reflex camera with a Thagee microscope attachment (Type 1). The magnification of the final photographs, which were obtained using Ilford FP 3 film and an exposure of 0.2 sec, was determined using a calibrated microscope slide.

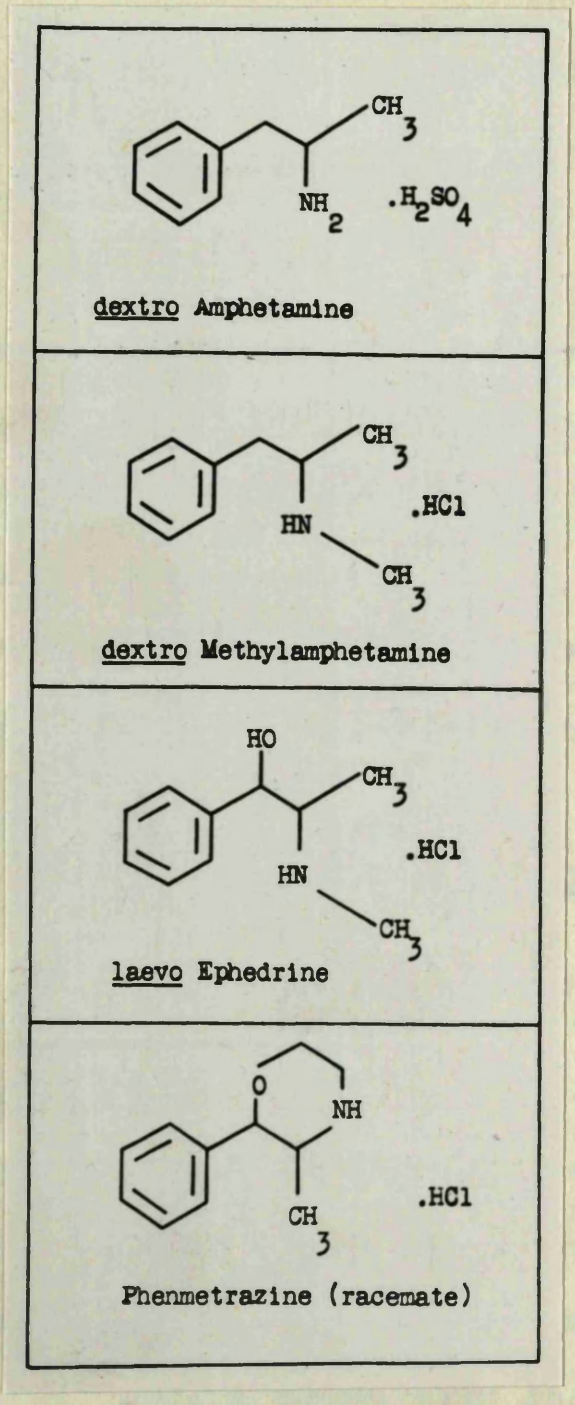

Fig. 49. Amphetamine and Related Phenylethylamine Derivatives.

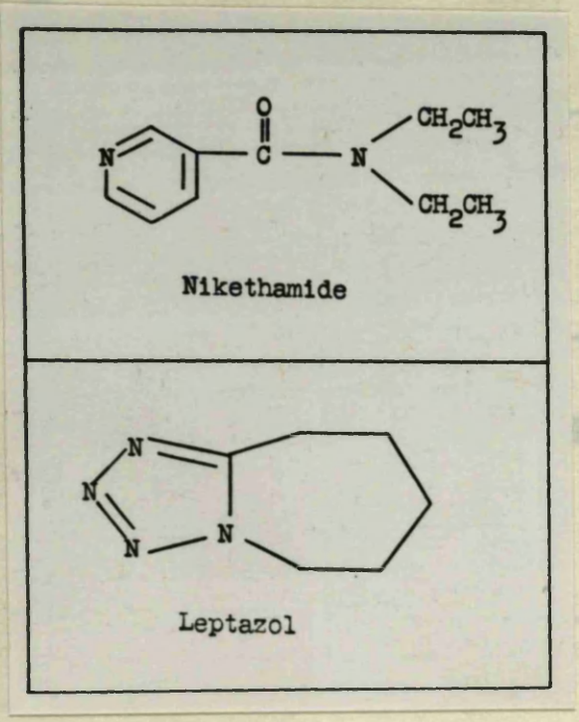

Fig. 51. Central Stimulants.

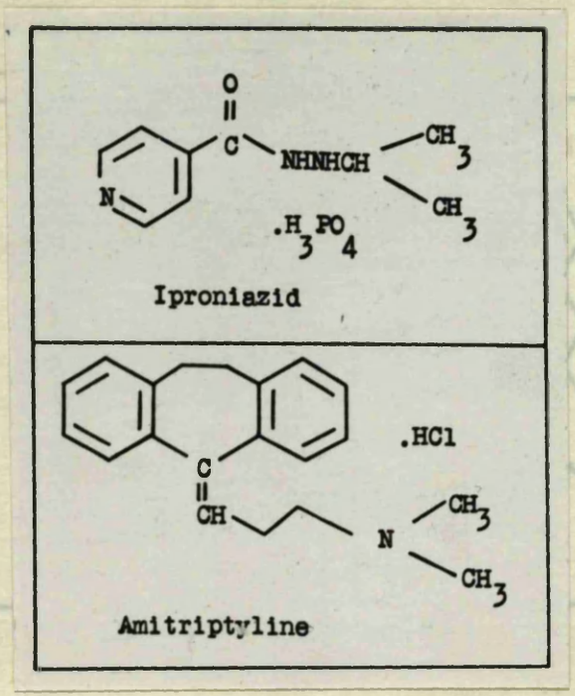

Fig. 50. Antidepressives.

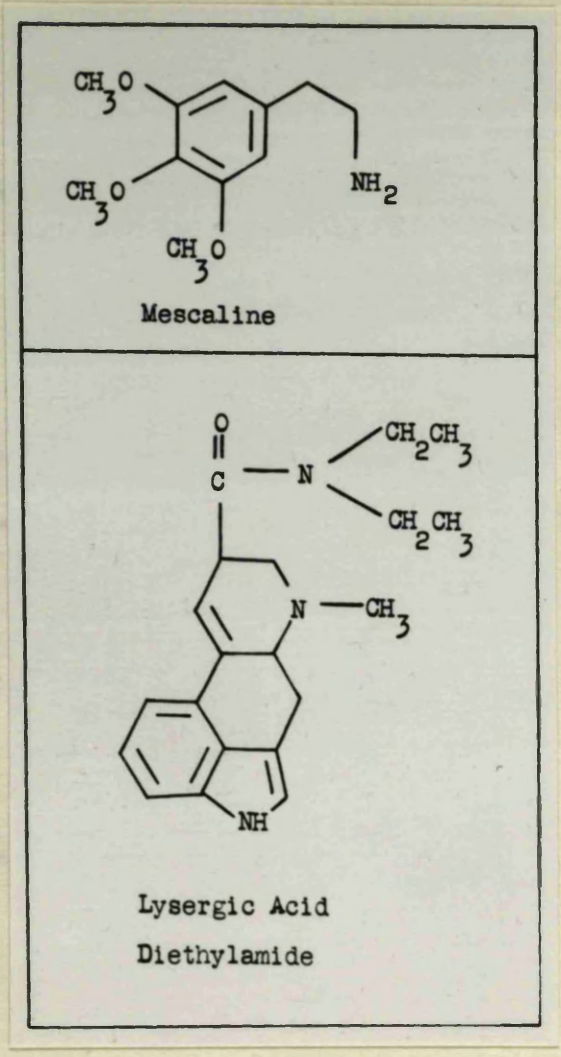

Fig. 53. Psychotomimetics.

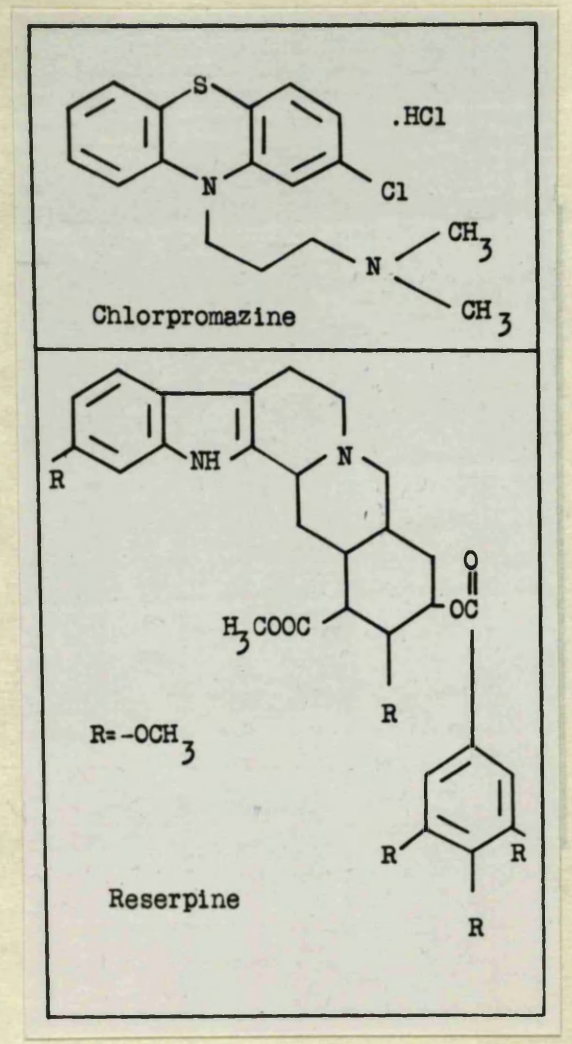

Fig. 52. Tranquillisers.

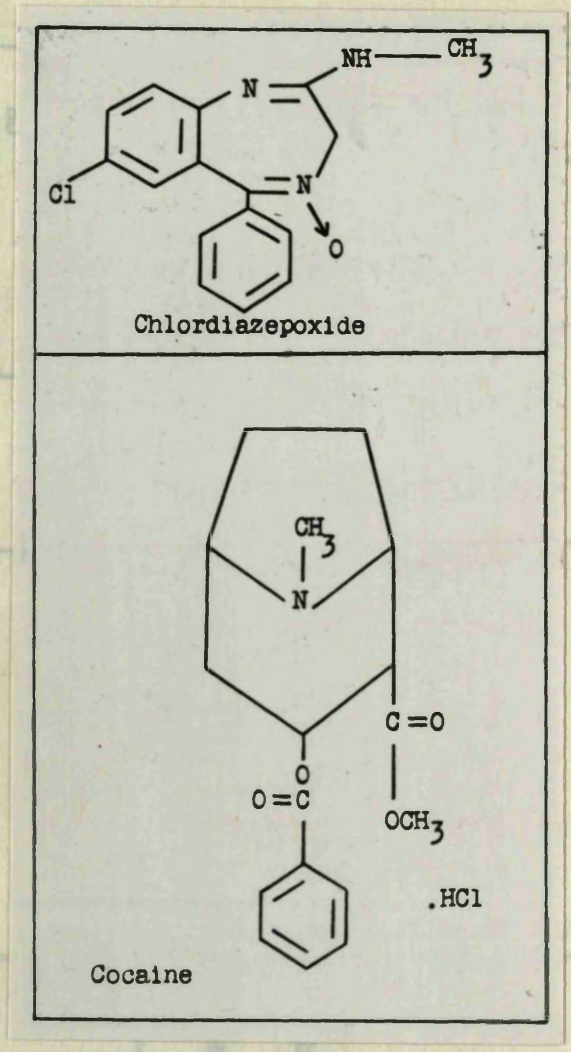

Fig. 54. Miscellaneous Drugs.

Preparation of Drug Solutions

The drugs (phenylethylamine derivatives, Fig. 49; antidepressives, Fig. 50; central stimulants, Fig. 51; tranquillisers, Fig. 52; psychotomimetics, Fig. 53; miscellaneous compounds, Fig. 54) were dissolved in 0.9% w/v sodium chloride solution so that the required dose was contained in 0.2 ml/100g of body weight. An equivalent volume of normal saline (0.9% w/v) was used in each experiment as a control.

Results

Table 4. Showing the Effects of Drugs upon Brain Nicotinamide Nucleotide Levels.

Series	Treatment	Dose mg/kg	No.	Time	Concentration (μM/g wet weight) NAD	$NADH_2$	NADP	$NADPH_2$
1	Amphetamine	2.5	9	3 hr	0.164 ± 0.010	0.020 ± 0.003	0.004 ± 0.001	0.017 ± 0.002
	Amphetamine	5	9		0.170 ± 0.012	0.021 ± 0.004	0.004 ± 0.001	0.011 ± 0.002
	Control	-	9		0.198 ± 0.011	0.020 ± 0.003	0.006 ± 0.001	0.015 ± 0.003
2	Chlorpromazine	15	9	3 hr	0.156 ± 0.009	0.021 ± 0.003	0.005 ± 0.001	0.012 ± 0.002
	Chlorpromazine	30	9		0.201 ± 0.006	0.023 ± 0.003	0.005 ± 0.001	0.013 ± 0.003
	Control	-	9		0.184 ± 0.010	0.023 ± 0.003	0.006 ± 0.001	0.011 ± 0.002
3	Amphetamine	2.5	10	2hr	0.176 ± 0.010	0.025 ± 0.004	0.007 ± 0.002	0.014 ± 0.002
	Control	-	10		0.191 ± 0.013	0.024 ± 0.003	0.005 ± 0.002	0.016 ± 0.002
4	Amphetamine	5	10	2hr	0.187 ± 0.006	0.030 ± 0.003	0.008 ± 0.003	0.016 ± 0.003
	Control	-	10		0.192 ± 0.005	0.029 ± 0.004	0.010 ± 0.006	0.018 ± 0.004
5	Amphetamine	10	10	2hr	0.163 ± 0.003	0.028 ± 0.004	0.005 ± 0.002	0.016 ± 0.003
	Control	-	10		0.198* ± 0.005	0.030 ± 0.004	0.017 ± 0.010	0.011 ± 0.002

6	Methyl-amphetamine	20	10	2 hr	0.173 ± 0.009	0.008 ± 0.001	0.009 ± 0.002	0.014 ± 0.003
	Control	-	10		0.199 ± 0.012 *	0.026 ± 0.009	0.013 ± 0.002	0.012 ± 0.001
7	Ephedrine	100	10	3 hr	0.201 ± 0.013	0.022 ± 0.007	0.005 ± 0.001	0.017 ± 0.004
	Control	-	10		0.192 ± 0.013	0.021 ± 0.006	0.009 ± 0.002	0.020 ± 0.004
8	Phenmetrazine	50	10	3 hr	0.193 ± 0.009	0.021 ± 0.006	0.011 ± 0.002	0.013 ± 0.002
	Control	-	10		0.195 ± 0.009	0.034 ± 0.010	0.014 ± 0.002	0.018 ± 0.004
9	Iproniazid	40	10	3 hr	0.189 ± 0.009	0.026 ± 0.004	0.007 ± 0.002	0.013 ± 0.002
	Control	-	10		0.183 ± 0.012	0.026 ± 0.004	0.012 ± 0.002	0.012 ± 0.002
10	Iproniazid	60	10	3 hr	0.198 ± 0.011	0.040 ± 0.010	0.014 ± 0.002	0.011 ± 0.003
	Control	-	10		0.202 ± 0.007	0.029 ± 0.010	0.008 ± 0.001	0.011 ± 0.002
11	Amitriptyline	50	10	3 hr	0.191 ± 0.004	0.038 ± 0.010	0.011 ± 0.003	0.013 ± 0.002
	Control	-	10		0.188 ± 0.006	0.027 ± 0.008	0.012 ± 0.002	0.008 ± 0.002
12	Niketha mide	250	10	2 hr	0.149 ± 0.018	0.034 ± 0.009	0.006 ± 0.002	0.015 ± 0.003
	Control	-	10		0.160 ± 0.016	0.023 ± 0.011	0.007 ± 0.002	0.011 ± 0.002

13	Leptazol	50	10	5 min	0.157* ± 0.003	0.041 ± 0.008	0.004 ± 0.001	0.011 ± 0.001
	Control	-	10		0.174 ± 0.007	0.036 ± 0.009	0.006 ± 0.001	0.009 ± 0.001
14	Chlorpromazine	40	10	2 hr	0.222 ± 0.007	0.031 ± 0.003	0.008 ± 0.002	0.015 ± 0.003
	Control	-			0.216 ± 0.008	0.027 ± 0.003	0.010 ± 0.001	0.015 ± 0.002
15	Reserpine	5	10	3 hr	0.224 ± 0.014	0.027 ± 0.004	0.008 ± 0.001	0.016 ± 0.004
	Control	-	10		0.216 ± 0.008	0.022 ± 0.004	0.010 ± 0.001	0.011 ± 0.002
16	Reserpine	10	10	3 hr	0.226 ± 0.037	0.031 ± 0.004	0.012 ± 0.004	0.015 ± 0.003
	Control	-	10		0.202 ± 0.040	0.026 ± 0.003	0.005 ± 0.001	0.013 ± 0.003
17	Reserpine	15	10	3½ hr	0.187 ± 0.007	0.022 ± 0.002	0.011 ± 0.002	0.015 ± 0.003
	Control	-	10		0.182 ± 0.012	0.027 ± 0.002	0.009 ± 0.001	0.011 ± 0.002
18	Mescaline	100	10	2 hr	0.176 ± 0.009	0.028 ± 0.002	0.004 ± 0.001	0.013 ± 0.002
	Control	-	10		0.206 ± 0.017	0.029 ± 0.002	0.004 ± 0.001	0.013 ± 0.001
19	Mescaline	150	9	2 hr	0.177 ± 0.010*	0.036 ± 0.008	0.010 ± 0.002	0.013 ± 0.002
	Control	-	9		0.213 ± 0.011	0.046 ± 0.009	0.009 ± 0.003	0.015 ± 0.002

20	LSD	0.10	10	1½hr	0.187 ± 0.014	0.022 ± 0.003	0.010 ± 0.002	0.014 ± 0.002
	Control	-	10		0.202 ± 0.009	0.024 ± 0.002	0.012 ± 0.003	0.016 ± 0.002
21	LSD	0.20	10	3 hr	0.165* ± 0.005	0.020 ± 0.005	0.006* ± 0.001	0.015 ± 0.002
	Control	-	10		0.194 ± 0.010	0.018 ± 0.005	0.014 ± 0.002	0.014 ± 0.003
22	Chlor-diazepoxide	100	10	3 hr	0.137 ± 0.007	0.022 ± 0.010	0.006 ± 0.002	0.011 ± 0.002
	Control	-	10		0.149 ± 0.004	0.018 ± 0.007	0.007 ± 0.002	0.009 ± 0.002
23	Cocaine	50	19	3 hr	0.166* ± 0.008	0.037 ± 0.010	0.006 ± 0.002	0.016 ± 0.002
	Control	-	19		0.187 ± 0.007	0.028 ± 0.006	0.009 ± 0.003	0.010 ± 0.002
24	Nicotinamide	250	10	3 hr	0.235* ± 0.009	0.045 ± 0.005	0.008 ± 0.001	0.017 ± 0.003
	Control	-	10		0.198 ± 0.010	0.038 ± 0.005	0.006 ± 0.002	0.009 ± 0.003

Table 4. Showing the Effects (± Standard Error of the Mean) of a Variety of Centrally-Acting Drugs upon the Oxidised and Reduced Nicotinamide Nucleotides of the Rat Brain. Results, Significantly Different from Control, are Marked by an Asterisk.

Effects of Drugs Upon Rat Brain Nicotinamide Nucleotide Levels

General Comments Table 4 shows the effects of a variety of centrally-acting drugs upon the levels of oxidised and reduced NAD and NADP in the rat brain. Throughout this investigation relatively few significant alterations in brain nicotinamide nucleotide levels were detected. NAD was generally found to be more susceptible to change than NADP, which was unaffected by most of the drugs investigated.

Effects of Amphetamine and Related Phenylethylamine derivatives In this group (Fig. 49) only the more potent central nervous system stimulants, amphetamine and methylamphetamine, produced any significant changes in brain NAD levels. The chemically and pharmacologically closely related drugs, ephedrine and phenmetrazine (Fig. 49), had no significant effects even at very high doses (100 mg/kg and 50 mg/kg respectively) (Fig. 56), which produced restlessness and an increased awareness of external stimuli.

Amphetamine had no effect at low dose levels

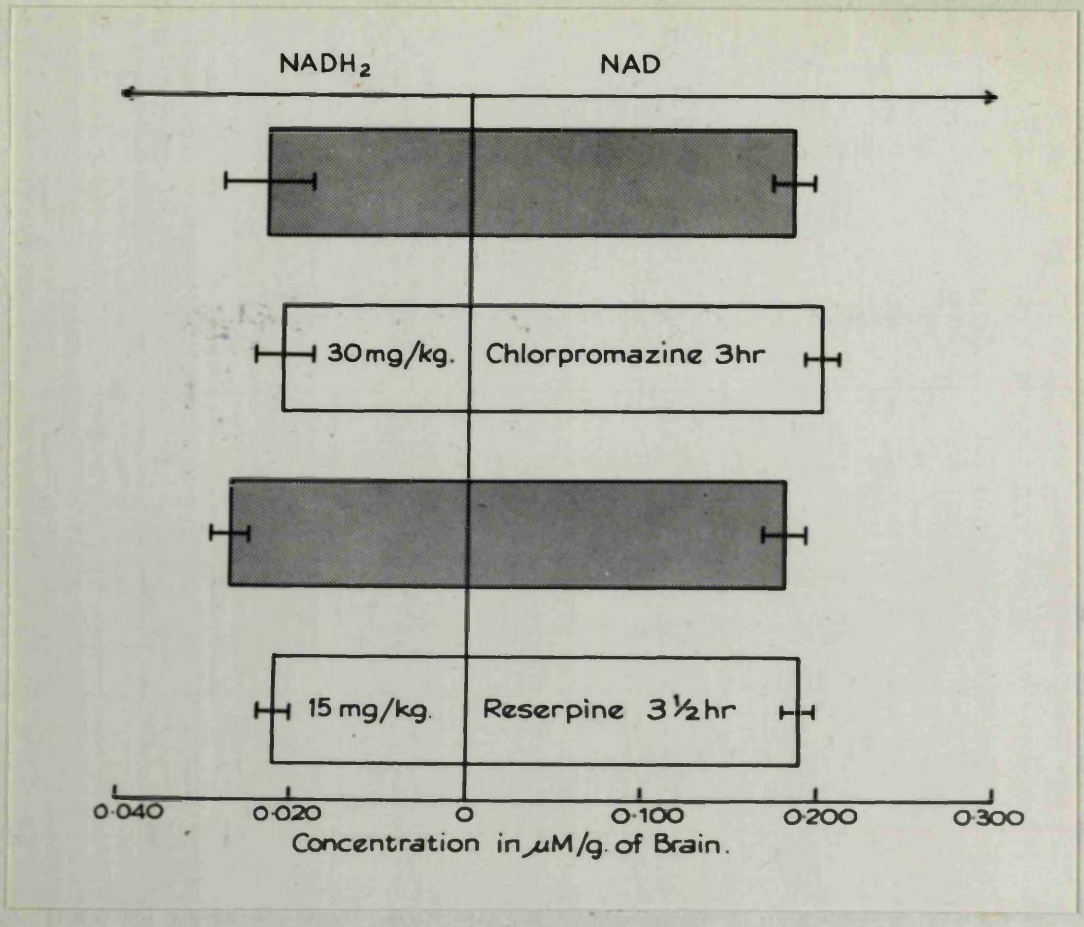
NADH2
NAD
30mg/kg.
Chlorpromazine 3hr
15 mg/kg.
Reserpine 3½hr
0·040
0·020
0
0·100
0·200
0·300
Concentration in μM/g. of Brain.

Fig. 55.

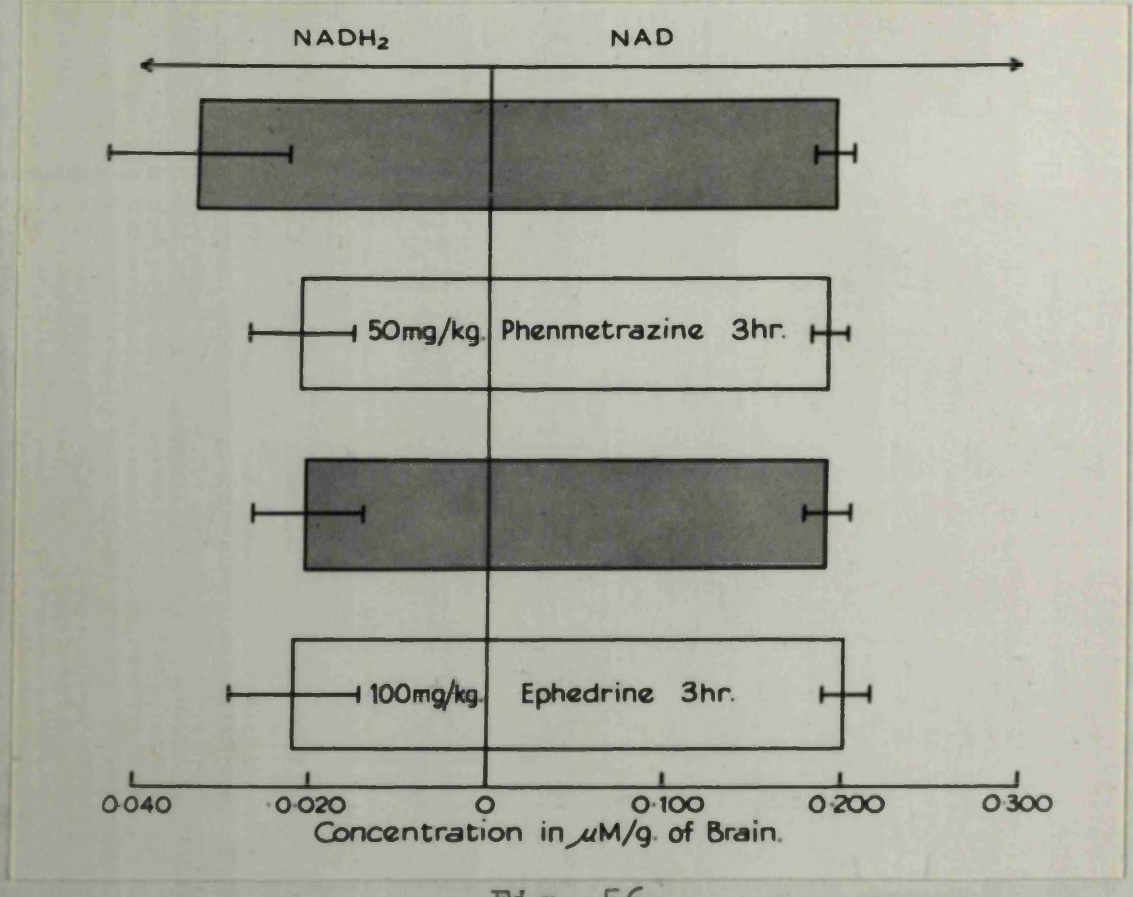
NADH2
NAD
50mg/kg.
Phenmetrazine 3hr.
100mg/kg.
Ephedrine 3hr.
0·040
0·020
0
0·100
0·200
0·300
Concentration in μM/g. of Brain.

Fig. 56.

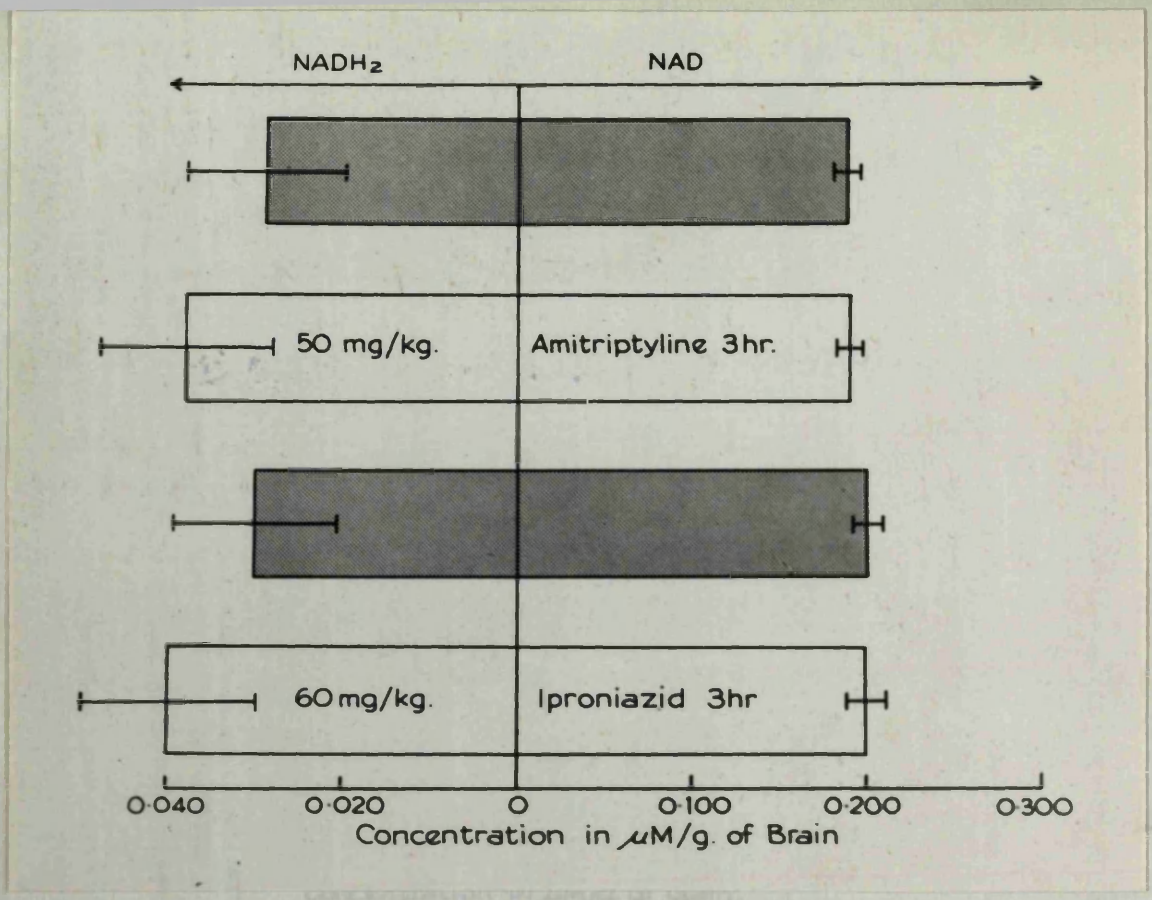
NADH2
NAD
50 mg/kg.
Amitriptyline 3hr.
60 mg/kg.
Iproniazid 3hr
0·040
0·020
0
0·100
0·200
0·300
Concentration in μM/g. of Brain

Fig. 57.

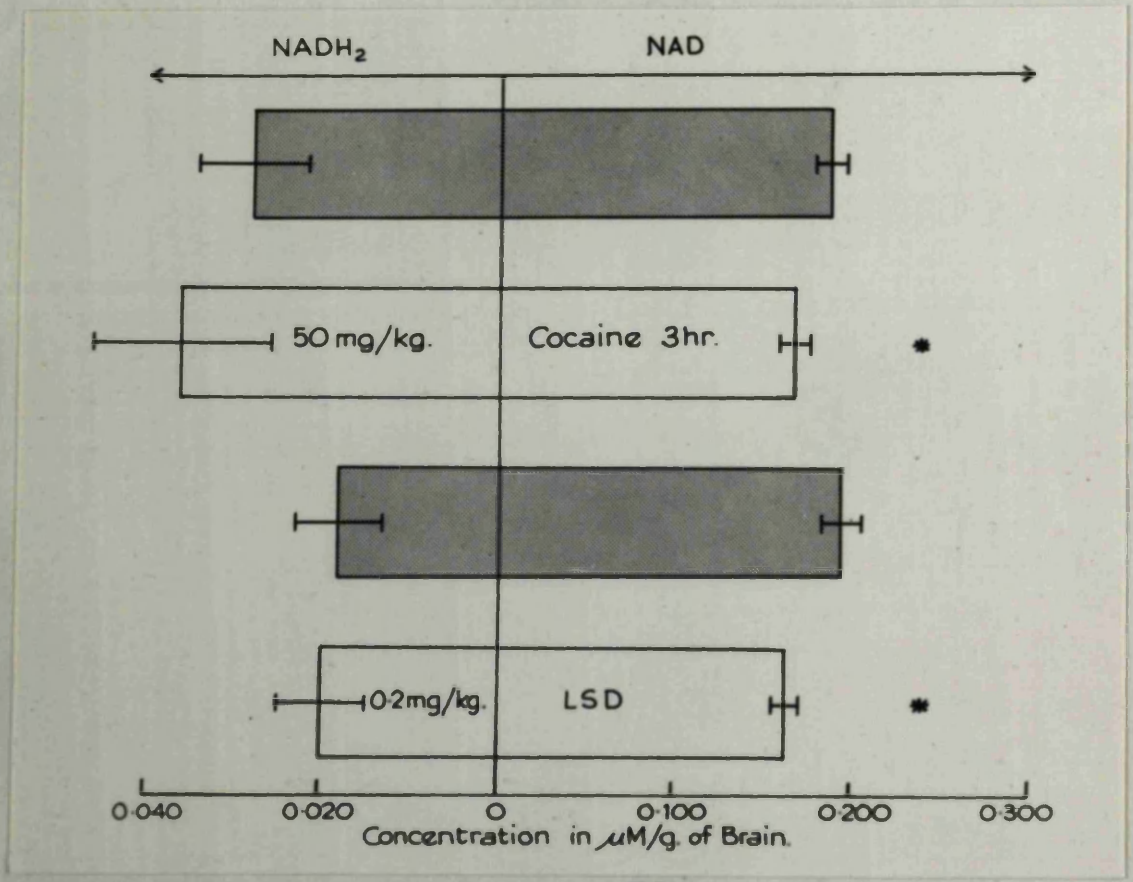
NADH2
NAD
50 mg/kg.
Cocaine 3hr.
0·2mg/kg.
LSD
0·040
0·020
0
0·100
0·200
0·300
Concentration in μM/g. of Brain.

Fig. 58.

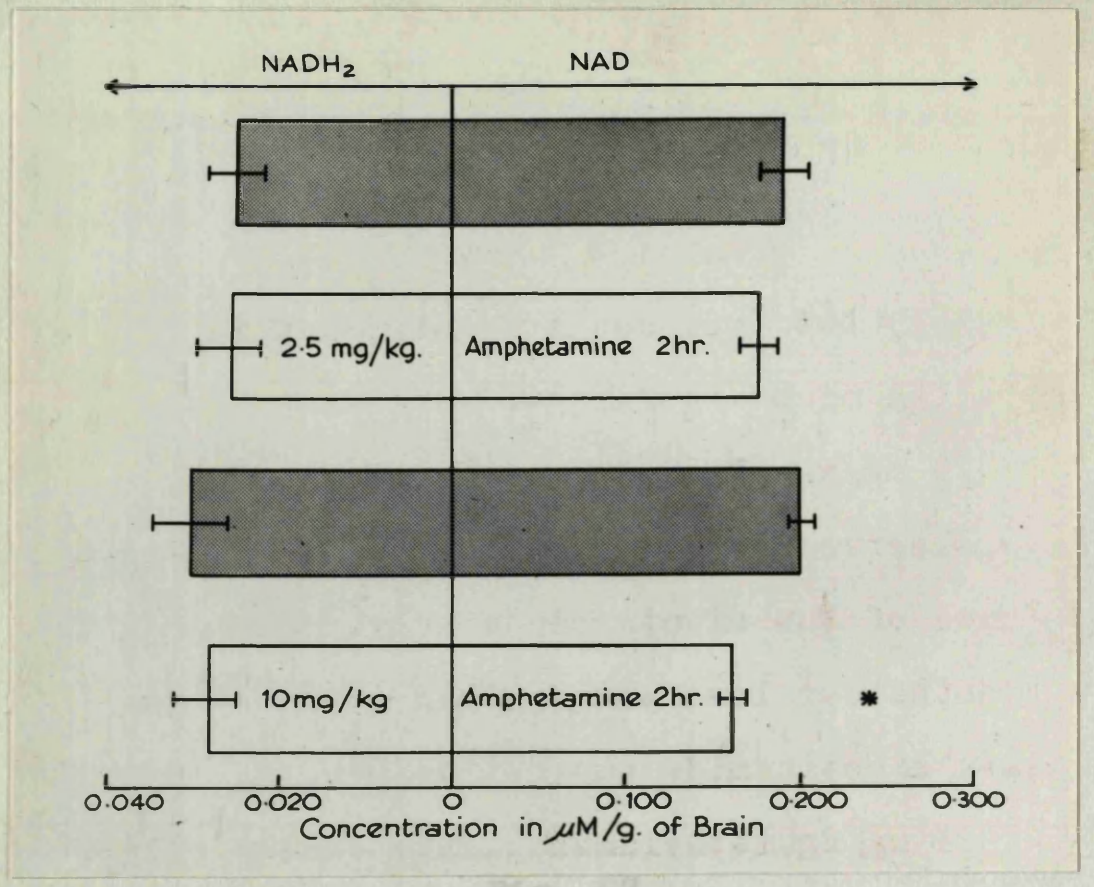
NADH2
NAD
2·5 mg/kg.
Amphetamine 2hr.
10mg/kg
Amphetamine 2hr.
*
0·040
0·020
0
0·100
0·200
0·300
Concentration in μM/g. of Brain

Fig. 59.

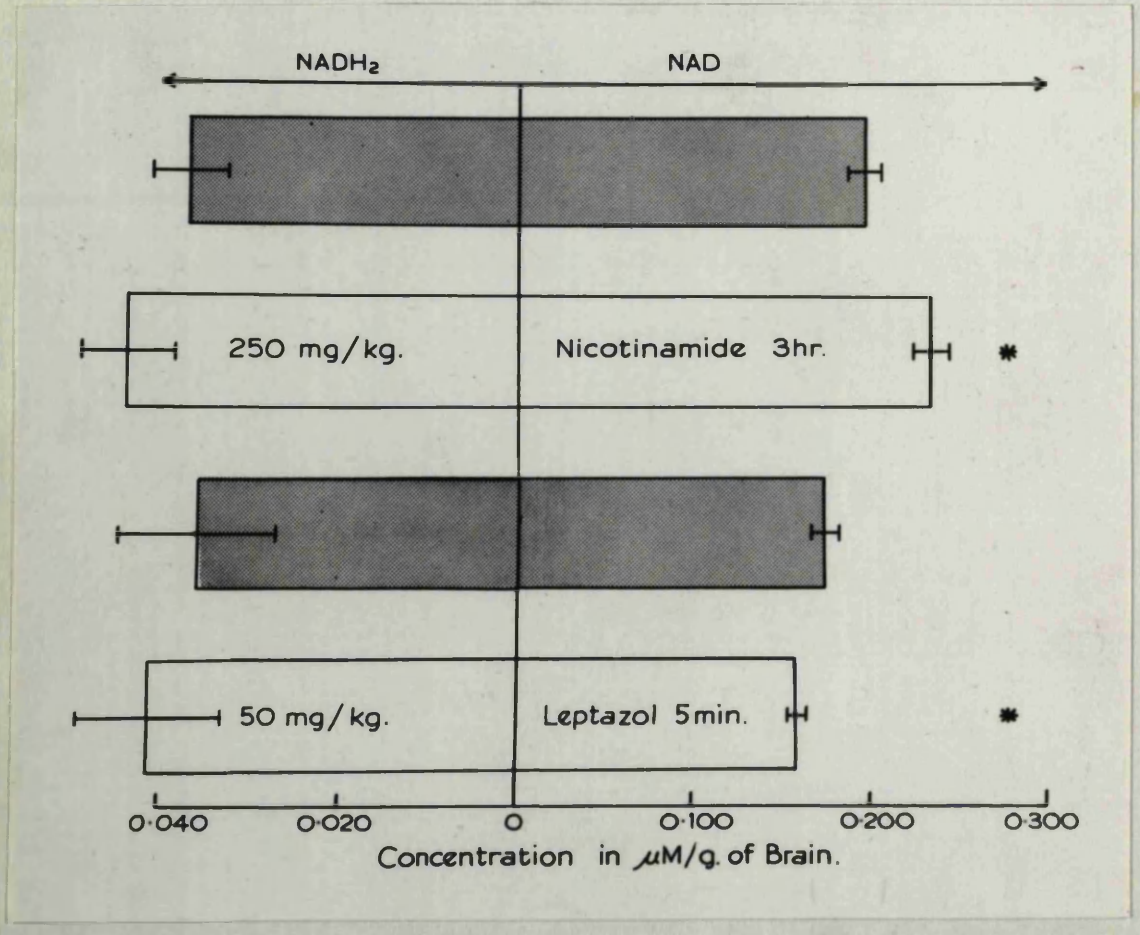
NADH2
NAD
250 mg/kg.
Nicotinamide 3hr.
*
50 mg/kg.
Leptazol 5min.
*
0·040
0·020
0
0·100
0·200
0·300
Concentration in μM/g. of Brain.

Fig. 60.

(2.5 and 5 mg/kg) but produced a significant fall in the concentration of brain NAD when the dose was increased to 10 mg/kg (Fig. 59). No corresponding changes were observed however, in the levels of any of the other forms of the nicotinamide nucleotides. Methylamphetamine was less potent than amphetamine in lowering brain nicotinamide nucleotide levels. Even at a dose of 20 mg/kg, methylamphetamine caused a fall only in the combined NAD and $NADH_2$ levels but did not affect either the oxidised or the reduced form individually.

Effects of Antidepressives Neither iproniazid (40 and 60 mg/kg) nor amitriptyline (50 mg/kg) (Fig. 50) produced any significant alterations in brain nicotinamide nucleotide levels (Fig. 57).

Effects of Central Stimulants The potent central nervous system stimulant, leptazol (50 mg/kg) (Fig. 51) caused a small but significant fall in brain NAD. This change occurred after 5 min, at a time when the animals were convulsing intermittently. None of the other forms of the nicotinamide nucleotides were affected (Fig. 60).

Nikethamide, on the other hand, had no significant effect at all upon brain nicotinamide nucleotide levels.

Effects of Tranquillisers Neither reserpine (5, 10 and 15 mg/kg) nor chlorpromazine (15, 30 and 40 mg/kg) (Fig. 52) affected brain nicotinamide nucleotide levels (Fig. 55), even when the animals showed signs of decreased locomotor activity and reduced response to external stimuli.

Effects of Psychotomimetics Both mescaline (150 mg/kg) and lysergic acid diethylamide (LSD) (0.2 mg/kg) (Fig. 53) lowered brain NAD levels. LSD was the more potent since it produced a significant reduction in both NAD and NADP at the remarkably low dose of 0.2 mg/kg (Fig. 58). Mescaline however, caused a fall only in the total NAD and $NADH_2$ content at the very high dose of 150 mg/kg. In this case NADP was unaffected.

Effects of Miscellaneous Compounds In this group (Fig. 54) only cocaine (50 mg/kg) produced a small though significant fall in brain NAD content (Fig. 58). Chlordiazepoxide (100 mg/kg) had no effect.

Table 4 also shows the effect of the important

Time Interval (hr)	Concentration of Brain NAD + NADP (μM/g, Wet Weight)									
	1		2		3		4		5	
Amphetamine	0.218 ±	0.006(6)	0.224 ±	0.002(7)	0.234 ±	0.005(9)	0.225 ±	0.005(6)	0.239 ±	0.008(6)
Control	0.217 ±	0.005(6)	0.192 ±	0.009(7)	0.176 ±	0.006(9)	0.209 ±	0.004(6)	0.226 ±	0.006(6)
P Value	-		0.001<P<0.01		P<0.001		0.05>P>0.02		-	
Cocaine	0.228 ±	0.004(8)	0.223 ±	0.005(8)	0.233 ±	0.006(8)	0.237 ±	0.005(10)	0.227 ±	0.003(5)
Control	0.220 ±	0.005(8)	0.201 ±	0.005(8)	0.188 ±	0.007(8)	0.176 ±	0.007(9)	0.218 ±	0.006(5)
P Value	-		0.001< P< 0.01		P< 0.001		P< 0.001		-	

Table 5. Showing the Effects (± Standard Error of Mean) of Amphetamine (10mg/kg) and Cocaine (50mg/kg) Upon the Total Oxidised Nicotinamide Nucleotide Content of Rat Brain at Different Time Intervals After Treatment. In Each Case the Number of Observations is Shown in Brackets.

	Treatment	Dose (mg/kg)	Concentration of NAD µM/g Tissue (wet weight)	
			Brain	Liver
(a)	Amphetamine	10	0.252 ± 0.033(5)	0.717 ± 0.096(6)
	Control	-	0.236 ± 0.027(5)	0.616 ± 0.072(6)
	Cocaine	50	0.269 ± 0.012(8)	0.742 ± 0.077(7)
	Control	-	0.267 ± 0.007(8)	0.851 ± 0.065(6)

	Treatment	Dose (mg/kg)	Counts (per min)/µM NAD/g Tissue (wet weight)	
			Brain	Liver
(b)	Amphetamine	10	4742 ± 1584(5)	5477 ± 1438(6)
	Control	-	1970 ± 106 (5)	9186 ± 1919(6)
	Cocaine	50	2249 ± 40 (8)	5035 ± 1261(7)
	Control	-	2080 ± 78 (8)	4039 ± 340 (6)

Table 6. Showing the Effects at 3 hr (± Standard Error of Mean) of Amphetamine and Cocaine upon (a) Brain and Liver NAD Levels and upon (b) the Incorporation of Nicotinamide (^{14}C) into NAD in Nicotinamide-Treated Rats. In Each Case the Number of Observations is Shown in Brackets.

NAD and NADP precursor, nicotinamide, which significantly increased brain NAD levels when administered at a dose level of 250 mg/kg.

Time Course Studies A number of experiments were carried out to determine the time course of the NAD-lowering actions of amphetamine (10 mg/kg) and cocaine (50 mg/kg). In this investigation, groups of animals, treated with either drug or control solution, were killed at different time intervals after injection and analyses were carried out of the total oxidised nicotinamide nucleotide contents of their brains (Table 5).

These studies, which were analysed using a simple t-test (Appendix III), revealed that amphetamine produced its maximum effect upon brain nicotinamide nucleotides at 3 hr, while cocaine exerted its maximum effect at 4 hr.

Radioactive Studies

These studies (Table 6) showed that the rate of incorporation of radioactive nicotinamide into both liver and brain NAD was not significantly affected either by amphetamine (10 mg/kg) or cocaine (50 mg/kg). They

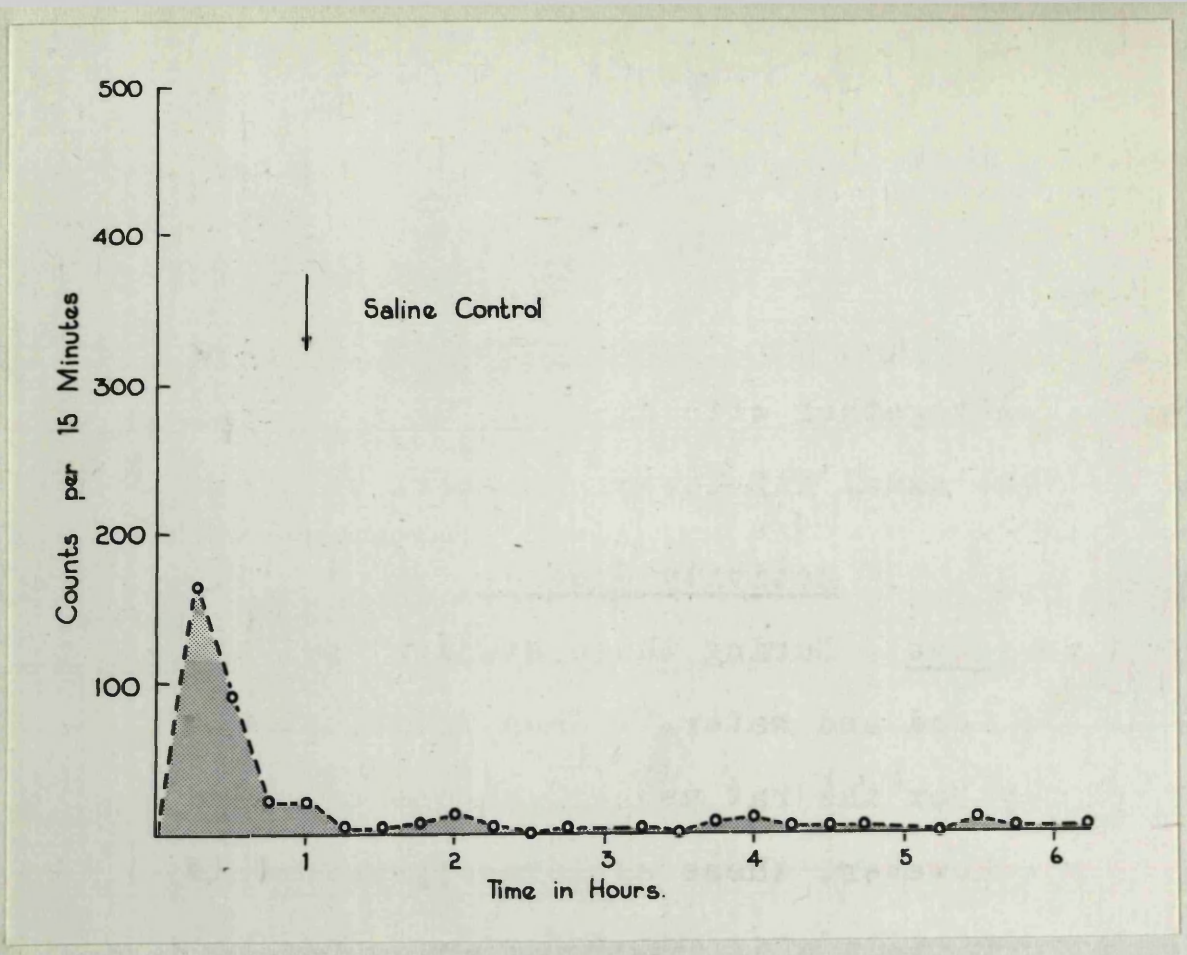
500
400
300
200
100
Counts per 15 Minutes
Saline Control
1
2
3
4
5
6
Time in Hours.

Fig. 61.

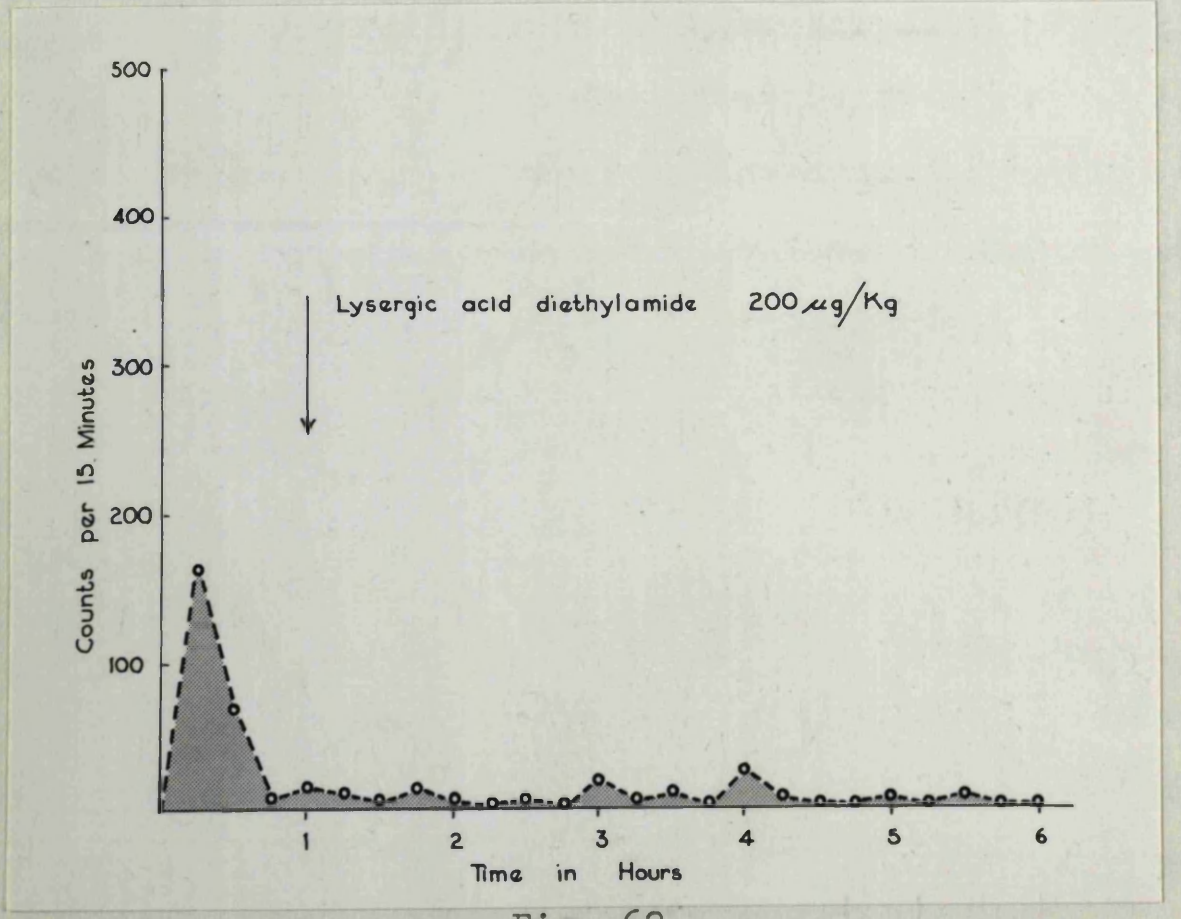
500
400
300
200
100
Counts per 15 Minutes
Lysergic acid diethylamide 200 µg/Kg
1
2
3
4
5
6
Time in Hours

Fig. 62.

also demonstrated that the administration of nicotinamide together with amphetamine or cocaine inhibited the usual NAD-lowering action of these drugs.

Activity Studies

General Comments During these studies the rats were deprived of food and water. When first introduced to the activity box the rat usually showed considerable activity. However, these exploratory movements gradually diminished towards the end of the first hour, when the rat, having completed investigating the new environment, settled down either to sleep or to continue with its normal maintenance operations. At the end of this preliminary control period, the rat was removed and injected with either the drug or control solution (Fig. 61). This interruption itself did not usually affect the rat's subsequent behaviour very much, as is indicated by the typical control record shown in Fig. 61. However, some of the drugs, which had been selected for their ability to lower brain NAD levels, did affect locomotor activity in the subsequent 5 hr.

Effects of LSD This drug (Fig. 62) (0.2 mg/kg) had no

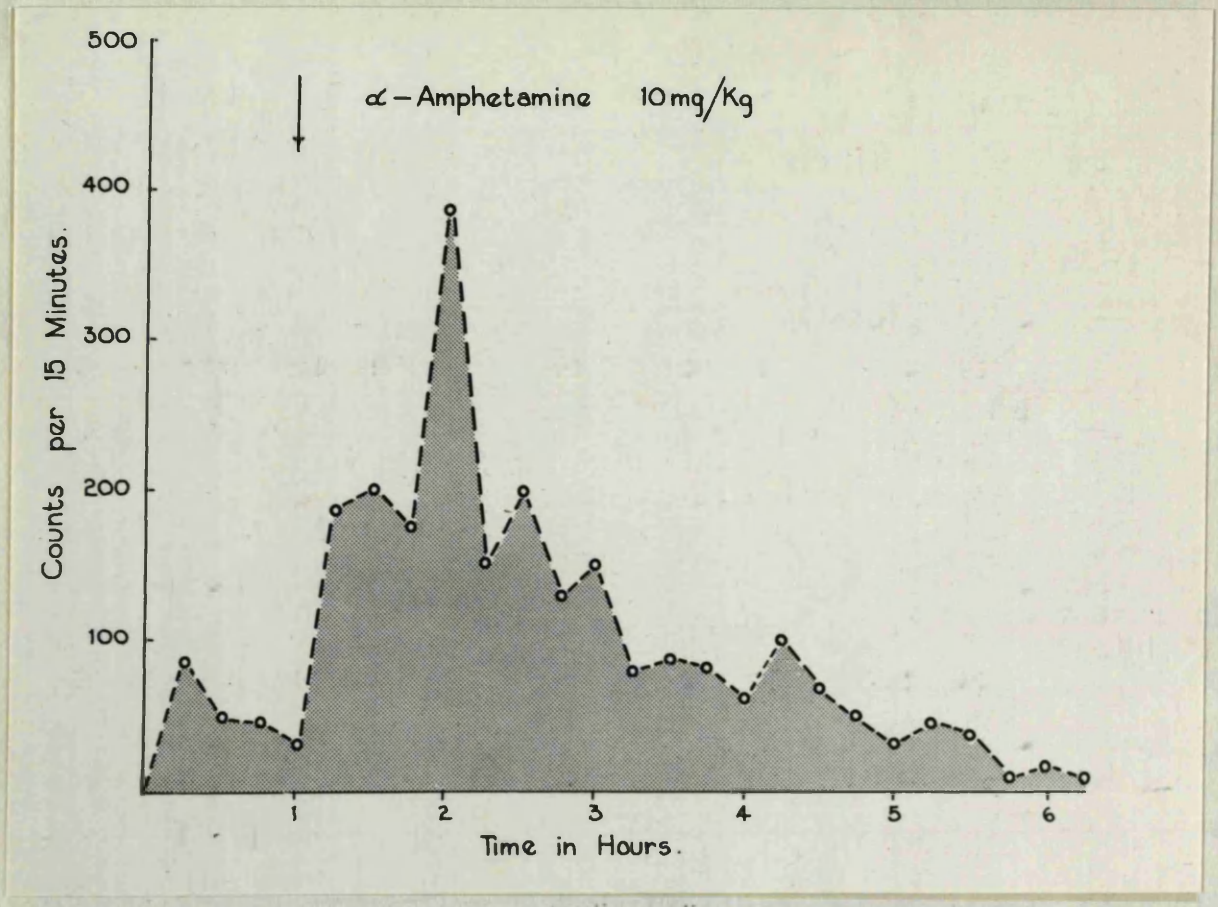
500
400
300
200
100
Counts per 15 Minutes.
α – Amphetamine 10 mg/Kg
1
2
3
4
5
6
Time in Hours.

Fig. 63.

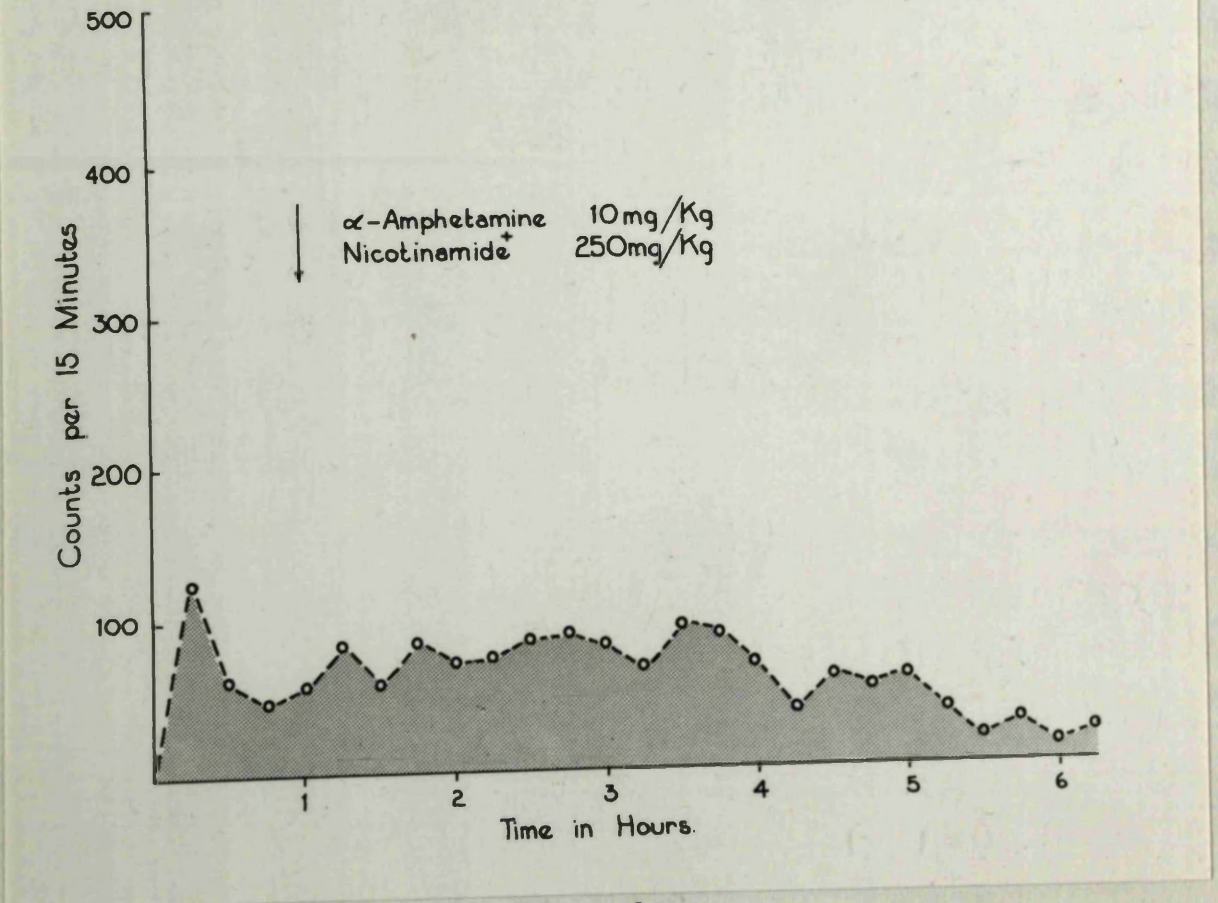
500
400
300
200
100
Counts per 15 Minutes
α-Amphetamine 10 mg/Kg
Nicotinamide 250 mg/Kg
1
2
3
4
5
6
Time in Hours.

Fig. 64.

	Time Interval (hr)	Counts/hr 1	2	3	4	5	6
(a)	Amphetamine	189 ± 20 (8)	228 ± 25 (10)	228 ± 26 (10)	47 ± 16 (8)	27 ± 13 (8)	16 ± 5(8)
	Control	171 ± 35 (8)	17 ± 4 (10)	6 ± 2 (10)	8 ± 3 (8)	11 ± 5 (8)	10 ± 3(8)
	P Value	-	P<0.001	P < 0.001	0.02<P<0.05	-	-
	Cocaine	233 ± 19 (8)	225 ± 29 (10)	180 ± 32 (9)	41 ± 12 (8)	14 ± 4 (8)	13 ± 4(8)
	Control	221 ± 23 (8)	20 ± 6 (10)	41 ± 16 (9)	20 ± 7 (8)	10 ± 6 (8)	11 ± 3(8)
	P Value	-	P < 0.001	0.001<P<0.01			
(b)	Amphetamine + Nicotinamide	227 ± 22 (10)	112 ± 15 (10)	107 ± 11 (10)	130 ± 19 (10)	71 ± 12(10)	37 ± 11(10)
	Amphetamine	188 ± 23 (10)	262 ± 35 (10)	129 ± 13 (10)	137 ± 23 (10)	73 ± 12(10)	32 ± 11(10)
	P Value	-	P < 0.001	-	-	-	-
	Cocaine + Nicotinamide	182 ± 28 (10)	270 ± 12 (10)	268 ± 24 (10)	238 ± 21 (10)	202 ± 16(10)	48 ± 11(10)
	Cocaine	187 ± 37 (10)	299 ± 22 (10)	257 ± 16 (10)	152 ± 22 (10)	103 ± 15(10)	40 ± 10(10)
	P Value	-	-	-	0.02>P>0.01	P<0.001	-

Table 7. Showing the Effects (± Standard Error of Mean) of (a) Amphetamine (10mg/kg) and Cocaine (50mg/kg) and (b) the Combination of these Drugs with Nicotinamide (250mg/kg) Upon Rat Locomotor Activity at Different Time Intervals. Drugs Were Administered at the End of the First Hour. In Each Case the Number of Observations is Shown in Brackets.

effect upon rat locomotor activity. Indeed, it produced a record, which was almost indistinguishable from the typical control (Fig. 61).

Effects of Amphetamine Amphetamine (Fig. 63) produced an almost immediate increase in motility. The very pronounced effect was sustained irregularly throughout the following 2 hr (Fig. 63). Separate confirmatory experiments, using statistical methods, were carried out to determine whether the apparent differences between drug and control at selected time intervals (1 hr, 2 hr, 3 hr, 4 hr and 5 hr after treatment) were significant. These studies (Table 7) showed that amphetamine significantly increased the locomotor activity 1, 2 and 3 hr after treatment. Maximum effects occurred after 1 and 2 hr. The lack of effect at the other time intervals reflects the gradual diminution in response and also the considerable variation, which is characteristic of this type of experiment.

When the rats were treated simultaneously with amphetamine (10 mg/kg) and nicotinamide (250 mg/kg) the

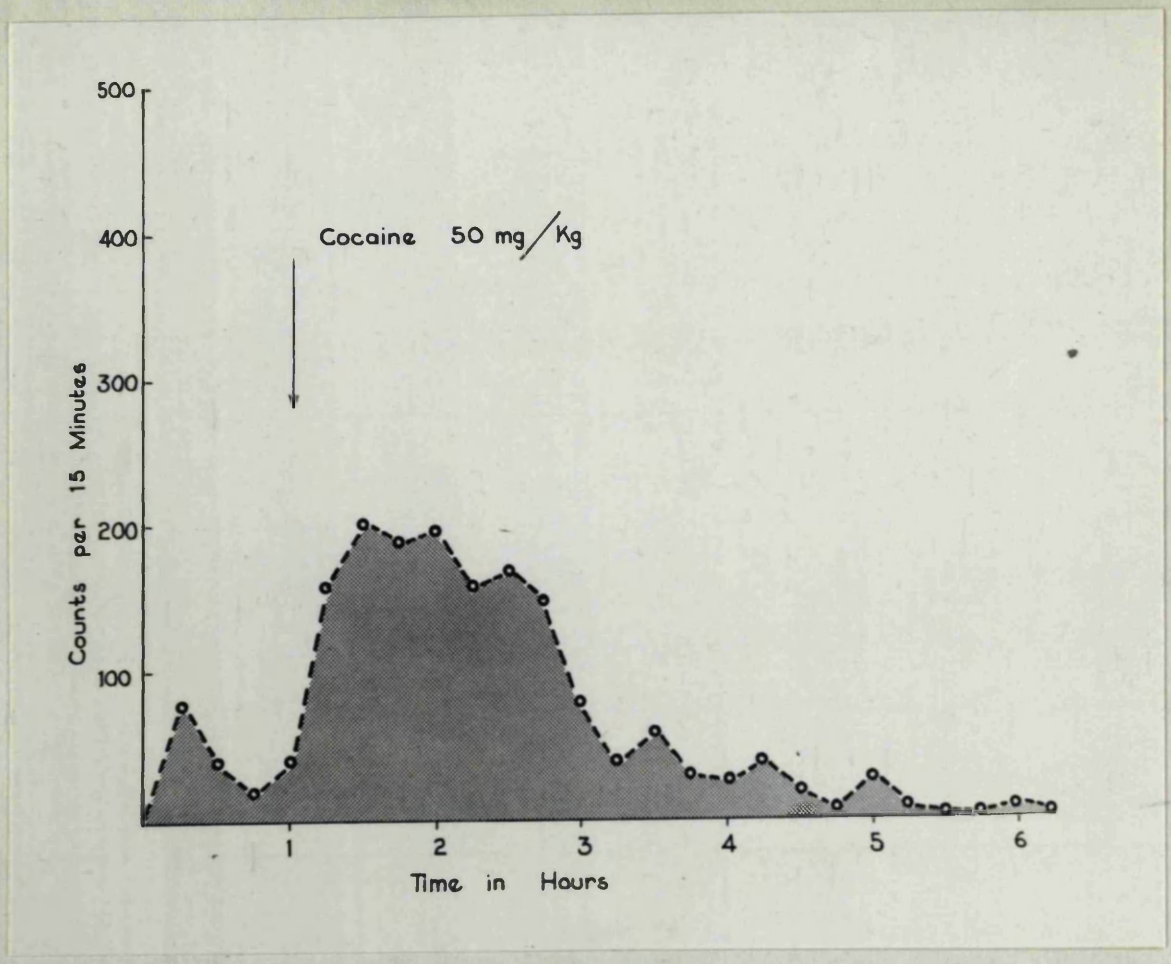
500
400
300
200
100
Counts per 15 Minutes
Cocaine 50 mg/Kg
1
2
3
4
5
6
Time in Hours

Fig. 65.

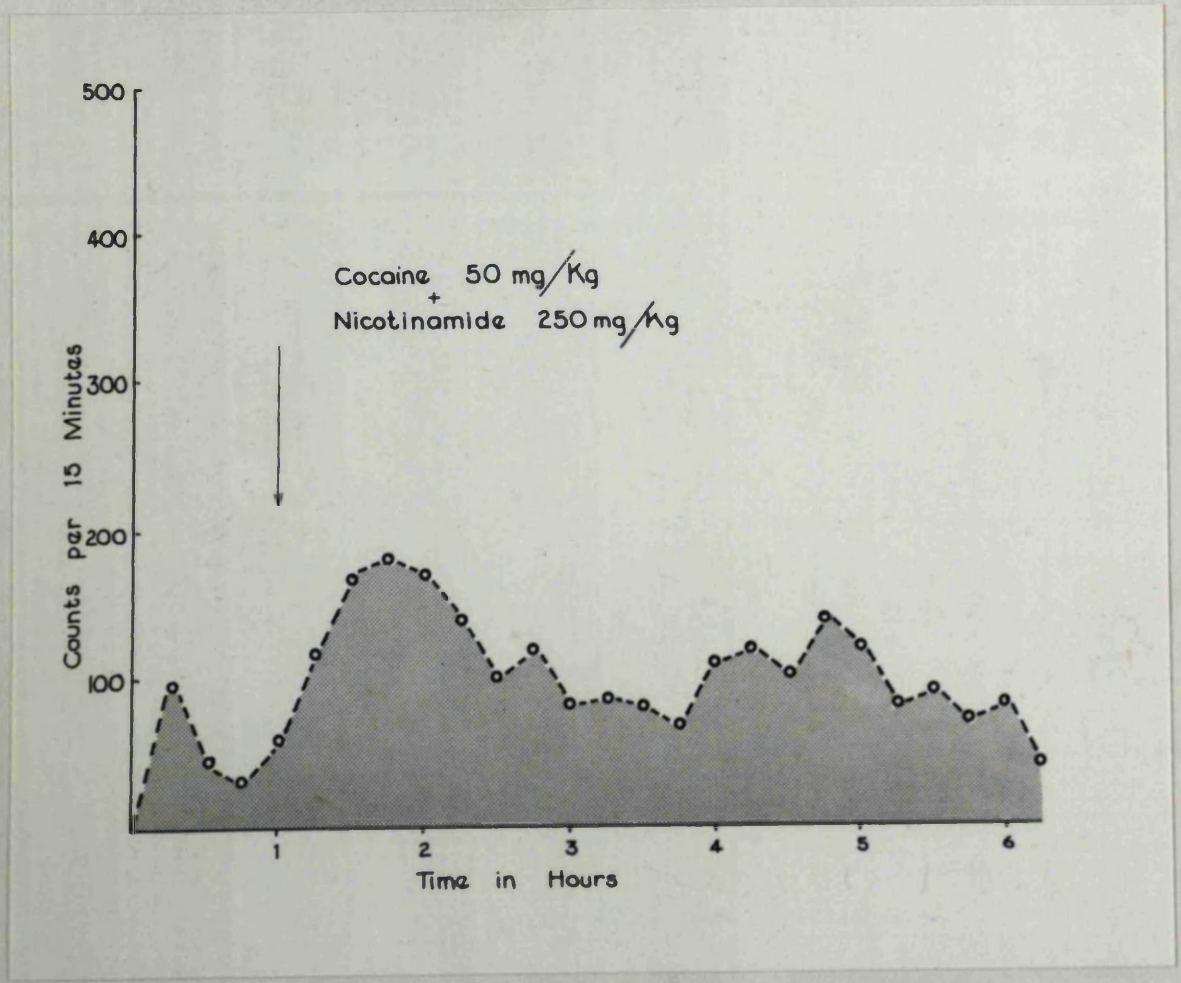
500
400
300
200
100
Counts per 15 Minutes
Cocaine 50 mg/Kg
+
Nicotinamide 250 mg/Kg
1
2
3
4
5
6
Time in Hours

Fig. 66.

locomotor activity increased only slightly above the normal control level (Fig. 64). At no time throughout the ensuing 5 hr did the activity level rise to the values, previously recorded for amphetamine alone (Fig. 63). This result was examined in a separate experiment, using statistical methods, which confirmed that the combined amphetamine-nicotinamide treatment produced a significant reduction in the locomotor response to amphetamine 1 hr after treatment.

Effect of Cocaine This drug (50 mg/kg) (Fig. 65) also increased the locomotor activity of the rats. The characteristic response again appeared very rapidly and was maintained for approximately 2 hr. Cocaine was less effective than amphetamine in stimulating motility but produced a less variable response. Statistical analysis of the differences between the control response and that of the cocaine-treated rats at selected time intervals showed that only the values 1 and 2 hr after treatment were significantly different. Again, the lack of effect at other time intervals probably reflects the great variation in the individual behavioural responses, as measured by this method.

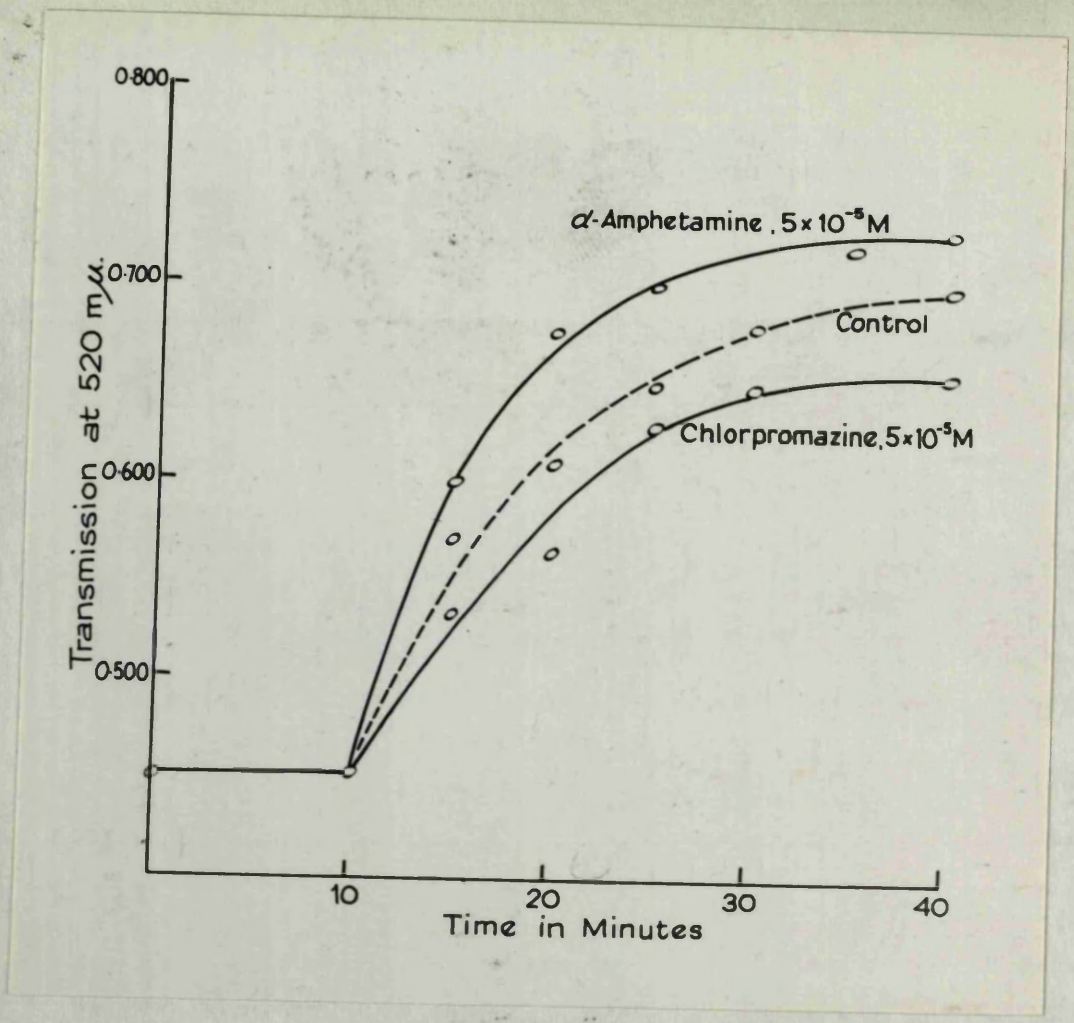
0·800
0·700
0·600
0·500
Transmission at 520 mμ.
α-Amphetamine . 5 × 10⁻⁵M
Control
Chlorpromazine. 5 × 10⁻⁵M
10
20
30
40
Time in Minutes

Fig. 67.

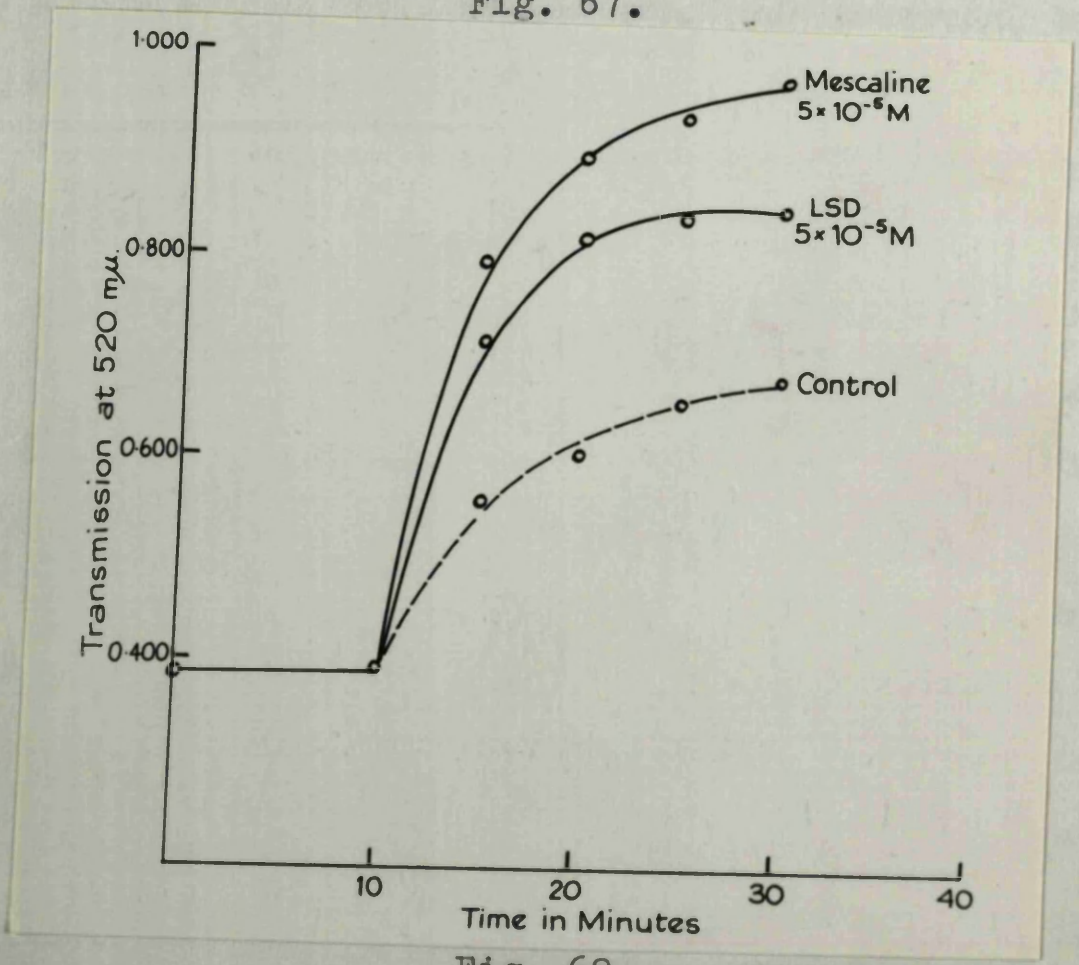
1·000
0·800
0·600
0·400
Transmission at 520 mμ.
Mescaline
5 × 10⁻⁵M
LSD
5 × 10⁻⁵M
Control
10
20
30
40
Time in Minutes

Fig. 68.

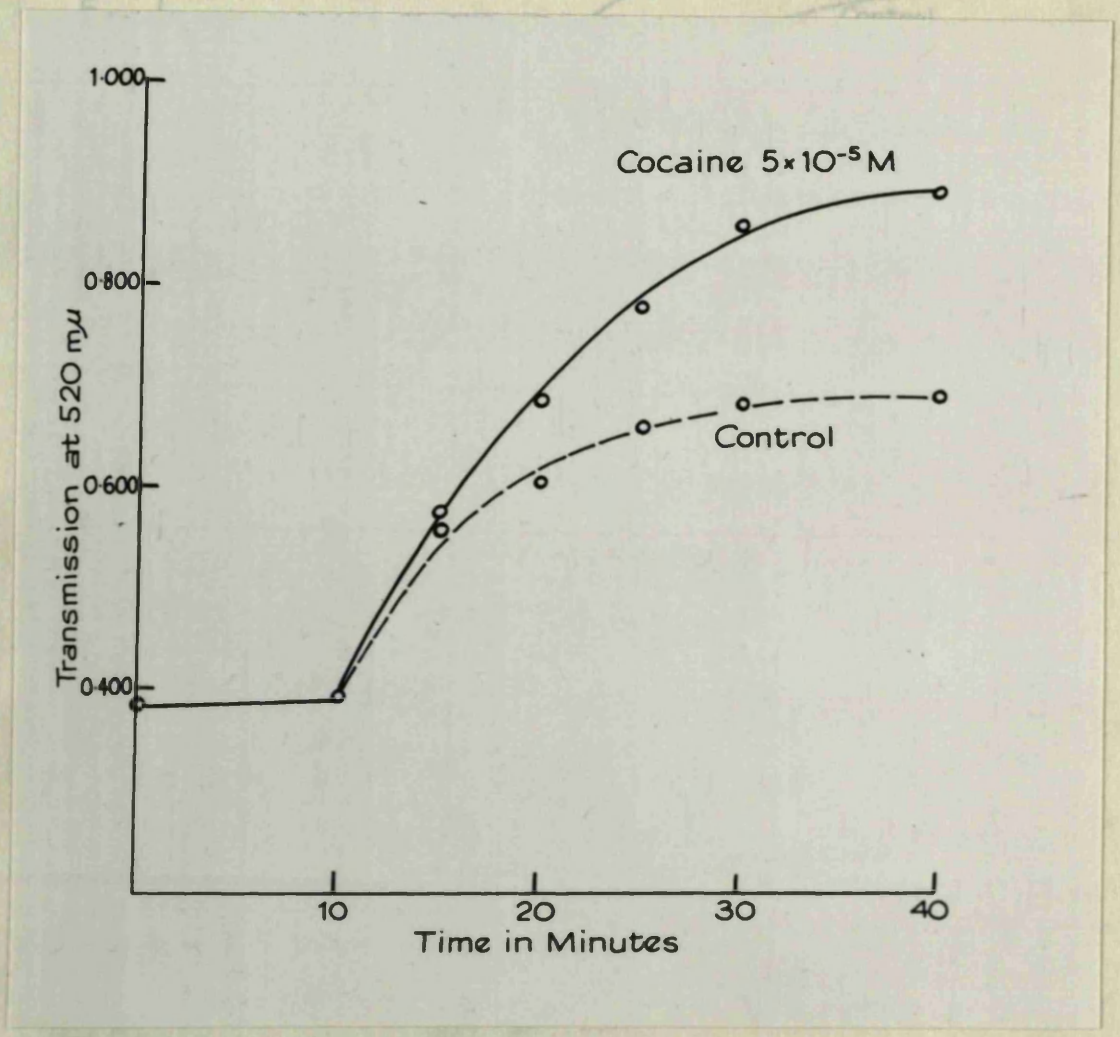
1·000
0·800
0·600
0·400
Transmission at 520 mμ
Cocaine 5×10⁻⁵M
Control
10
20
30
40
Time in Minutes

Fig. 69.

The combination of cocaine (50 mg/kg) and nicotinamide (250 mg/kg) produced a response, which was different from that of cocaine alone. The initial locomotor-stimulating effect was similar to that of cocaine but was less pronounced. However, 4 and 5 hr after treatment with the drug combination, the motility increased or at least did not diminish in the usual manner (Fig. 66). This response was confirmed by a separate experiment (Table 7), which showed that cocaine and nicotinamide together produced statistically significant increases in locomotor activity 3 and 4 hr after treatment, when compared with the effect of cocaine alone (Table 7).

Mitochondrial Swelling Studies

Spectrophotometric Method The results of the indirect spectrophotometric method of measuring mitochondrial swelling are shown in Figs. 67, 68 and 69. These indicate that the gradual increase in the transmission (i.e. reduction in optical density), which occurred in the mitochondrial suspension in these circumstances, could be accelerated by some of the drugs, which reduced brain NAD levels. Thus, amphetamine, mescaline,

Treatment	Dose	Optical Density	P Value
Amphetamine	4×10^{-4}M	0.169 ± 0.012 (9)	0.001<P<0.01
Control	-	0.223 ± 0.010	
Amphetamine	4×10^{-5}M	0.188 ± 0.016 (9)	0.025<P<0.05
Control	-	0.219 - 0.009 (9)	
Cocaine	4×10^{-5}M	0.148 ± 0.017 (7)	0.001<P<0.01
Control	-	0.198 ± 0.016 (7)	
Mescaline	4×10^{-4}M	0.172 ± 0.022 (9)	0.025<P<0.05
Control	-	0.230 ± 0.024 (9)	
Chlorpromazine	4×10^{-4}M	0.239 ± 0.014 (9)	0.001<P<0.01
Control	-	0.178 ± 0.020 (9)	

Table 8. Showing the Effects (± Standard Error of Mean) of Drugs Upon the Optical Density of a Suspension of Rat Brain Mitochondria Incubated (10 min) In Vitro. In Each Case the Number of Observations is Shown in Brackets.

LSD and cocaine increased transmission at 520 mμ, when present at a concentration of 5×10^{-5}M. On the other hand, chlorpromazine (5×10^{-5}M) inhibited this reponse.

Separate experiments, using statistical methods confirmed the graphical results (Figs. 67, 68 and 69) by showing (Table 8) that amphetamine, mescaline and cocaine significantly increased, whereas chlorpromazine decreased transmission in mitochondrial suspensions.

Gravimetric Method The spectrophotometric method depends upon measuring the fall in optical density, which accompanies the influx of water into the mitochondria during swelling. This indirect technique lacks specificity, since it does not discriminate between mitochondrial swelling and any other process, which might produce an increase in transmission in the suspension. On the other hand, the gravimetric method depends upon measuring the water content directly and therefore gives a more satisfactory estimate of the effects of drugs upon mitochondrial volume.

In these experiments (Table 9) amphetamine,

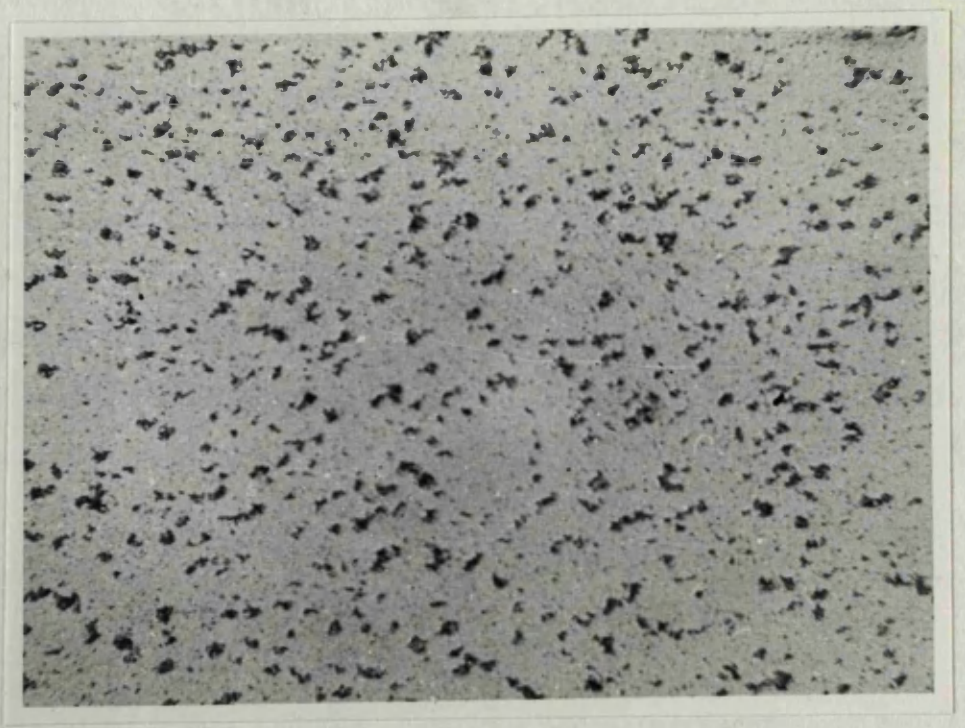

Fig. 70.

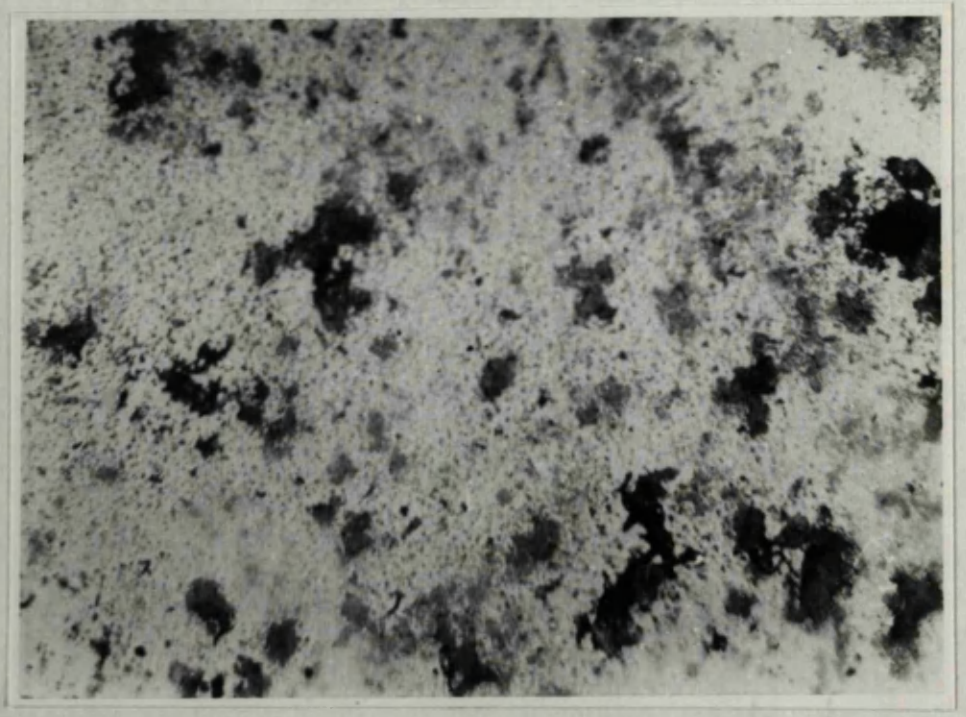

Fig. 71.

cocaine and mescaline had no significant effects upon the water content (percentage weight) of the mitochondria. These results suggested that the effects observed in the previous experiment were merely the reflection of mitochondrial aggregation rather than of swelling. This interpretation was confirmed by microscopy, which demonstrated clearly that amphetamine, cocaine and mescaline caused pronounced clumping of the mitochondria. A typical example of this phenomenon is given in Figs. 70 and 71, which illustrate respectively a control suspension (Fig. 70) and the effect of amphetamine (5×10^{-4}M, 10 min) (Fig. 71) upon rat brain mitochondria <u>in vitro</u> (magnification x 400).

Summary of Results

A variety of centrally-acting drugs, including the stimulants amphetamine and cocaine, the convulsant leptazol and the psychotomimetics mescaline and LSD, significantly lowered brain nicotinamide nucleotide levels. In addition, amphetamine and cocaine increased locomotor activity. However, these two responses were not directly related, since the time courses of the locomotor-stimulant effects did not parallel the NAD lowering actions. The lack of correlation between the behavioural and the biochemical responses is further emphasised by LSD, which lowered brain NAD levels but did not increase motility. On the other hand, the locomotor effects of amphetamine and cocaine were modified by the NAD precursor, nicotinamide, which reduced the locomotor response to amphetamine but prolonged that of cocaine.

Certain central nervous stimulants and psychotomimetics, which lowered brain NAD levels also apparently accelerated spectrophotometrically-determined mitochondrial swelling. The discrepancy between this result and the lack of effect observed in gravimetric

measurements of mitochondrial swelling were resolved by microscopy, which revealed that these drugs caused *in vitro* aggregation.

Attempts to correlate the drug-induced reduction in brain nicotinamide nucleotide levels with alterations in the rate of incorporation of radioactive nicotinamide into the pyridine coenzymes, revealed that this reaction was not significantly affected by either amphetamine or cocaine. These studies also showed that the simultaneous administration of nicotinamide with either amphetamine or cocaine prevented the usual NAD-lowering response to either of the latter drugs.

Discussion

Discussion of Methods

The experimental methods used to measure biological responses may profoundly influence the results obtained. This is true whether the parameter being investigated is a labile nucleotide ($NADH_2$) or a particular aspect of behaviour (locomotor activity).

Nicotinamide Nucleotide Assay The nucleotides are susceptible to enzymic oxidation ($NADH_2 \longrightarrow NAD$) and hydrolysis (by NADase). In measuring the brain levels of these coenzymes it is therefore necessary to inhibit those enzyme-catalysed reaction, which might continue to operate after death. The pyridine coenzymes present special difficulties, since they exist in both oxidised and reduced forms, which are destroyed by alkali and acid respectively. As a result, it is impossible to inactivate the hydrolytic and oxidative enzymes and at the same time, extract all the nicotinamide nucleotides, using such common protein precipitants as perchloric acid and

trichloracetic acid, which would destroy the reduced coenzymes. In these circumstances, it is necessary to prepare a second tissue extract using strong alkali, in which the reduced coenzymes are comparatively stable. This may be combined with a liquid nitrogen freezing technique for killing the animals. However, it introduces the major difficulty of having to divide the brain into two equal and anatomically-similar parts. The need for this arises from the heterogeneity of the brain and the uneven distribution of NADase. An alternative method, using hot TRIS buffer both to extract the oxidised and reduced nicotinamide nucleotides and to inactivate the associated enzymes was preferred. The possibility of combining this procedure with killing the animals by the freezing technique was investigated and found to be unsatisfactory. The almost complete disappearance of the reduced nucleotides (NAD+NADP, $0.187 \pm 0.029 \mu M/g$; $NADH_2+NADPH_2$, $0.003 \pm 0.002 \mu M/g$) suggests that the freezing and subsequent thawing of the brain in hot TRIS buffer may have activated or released such

Reference	Number of Observations	Concentration of Nucleotide (μM/g of Brain)			
		NAD	$NADH_2$	NADP	$NADPH_2$
1	3	0.200 ± 0.009	0.133 ± 0.054	0.003	0.012 ± 0.004
2	3	0.241	0.107	0.010	0.012

Table 10. Showing the Concentrations of NAD, $NADH_2$, NADP and $NADPH_2$ in Rat Brain as Measured by (1) Glock et al, 1955 and (2) Lowry et al, 1957.

enzmes as NAD-cytochrome c reductase and NADase (Cunningham, Crane & Sottocasa, 1965; Decsi, 1965).

Strict comparisons cannot be made between the results obtained in different laboratories because of the variations in age, sex and weight of the experimental animals, (Burton, 1957) in addition to the more important differences in the extraction and analytical procedures. Nevertheless, the results obtained for the oxidised nucleotides and $NADPH_2$ in the present investigation (Table 4) agree with those of other workers (Table 10). However, the $NADH_2$ levels are lower than those obtained in other investigations. This may be due to oxidation of the reduced nucleotides, either during the hot TRIS buffer extraction of the brain or, in some cases, as a result of storing the frozen extracts at $-15^{\circ}C$ and thawing them just prior to the assay (Pande, Bhan & Venkitasubramanian, 1964). On the other hand, there is no evidence that low brain $NADH_2$ levels are associated with high NAD levels.

In this investigation, the experimental error

involved in assaying the tissue nicotinamide nucleotides was small for NAD but large for NADP. This marked difference reflects the great difficulties inevitably associated with measuring very small quantities of labile material in the brain. It is possible that these large errors in the case of NADP and $NADPH_2$, and to some extent also in the case of $NADH_2$, may have masked drug-induced changes, which might only have become apparent had very large numbers of animals been used.

Since fluorescence may be affected by interfering substances it is necessary to determine whether the apparent reduction in brain nicotinamide nucleotide levels might not simply be due to quenching, either by the drug itself or by some substance mobilised by the drug within the animal (e.g. glucose). These possibilities are unlikely, since drugs which lowered brain NAD levels did not necessarily affect all the other forms of the nicotinamide nucleotides in the same manner. Furthermore, the use in each assay of separate internal reference standards, consisting of

either NAD or NADP, dissolved in a tissue blank, eliminated this risk entirely.

Activity Studies Locomotor studies provide very limited information about the central nervous system effects of psychotropic drugs. Such experiments measure a gross response, which is the resultant of a multitude of biochemical and physiological events. This parameter is therefore subject to great variation and may be modified by many factors.

Generally, an increased locomotor response may be associated with increased central nervous system activity. The converse is not necessarily true. For example, animals may become agitated and show many signs of excitement including repetitive head movements or an excessive interest in cleaning operations. In these circumstances the animal may move about its environment very little. Such signs of central nervous system stimulant activity would not necessarily be registered by the conventional photo-electric cell activity box, used in this investigation but could only be recorded by more sensitive devices

(Knoll, 1961; van Rossum, van Ameron, Daamen, Hurkmans, Megens & Peters, 1962).

Mitochondrial Studies The basic assumption underlying all *in vitro* studies is that the biochemical reactions and physiological mechanisms occurring in the intact animal also take place to some extent in isolated tissues, homogenates and subcellular particles. Despite the undoubted value of these studies, especially in the field of drug-enzyme interactions, it is necessary to interpret all *in vitro* experiments with caution and to regard hypotheses based upon them with scepticism. Before the *in vivo* pharmacodynamic activity of a drug can be explained on the basis of a particular *in vitro* effect it is necessary to establish whether or not the same mechanism is affected *in vivo* and whether such an interference would qualitatively and quantitatively explain the action of the drug. In assessing the relevance of *in vitro* experiments it is essential to compare the concentration of drug producing the *in vitro* effect with that required to produce the *in vivo* response. The inability of a drug to affect an *in vitro* mechanism does not prove conclusively that the mechanism

is unaffected *in vivo*.

In vitro studies on mitochondrial swelling are very difficult to interpret. The optical method is unsatisfactory, since it reflects a variety of disparate processes, including swelling, aggregation, fragmentation and dissolution of the mitochondria (Robinson, 1964). The gravimetric method may also be criticised, since centrifugation could "squeeze" water from the swollen mitochondria.

Discussion

General Comments Centrally-acting drugs probably influence, either directly or indirectly, an enormous number of physiological and biochemical mechanisms within the brain. It is therefore very difficult to distinguish between cause and effect and to identify the principal pharmacodynamic activity of each drug. Small, seemingly insignificant reactions, could affect more prominent mechanisms, which might in turn overshadow the primary interaction. For example, during nerve cell depolarisation the following closely integrated reaction sequence might occur

> glycolysis - electron transport
> - oxidative phosphorylation -
> - synthesis, storage and release
> of acetylcholine - changes in
> membrane permeability - ion
> movements - depolarisation (Decsi, 1965).

In this case, slight drug-induced changes in nicotinamide metabolism could affect glycolysis in the cytoplasm and the electron transport chain in the mitochondria. Thus, even minor alterations in the

relative levels of these nucleotides could ultimately be manifested in proportionately large changes in the acetylcholine content of the cell. Obviously, different types of drugs could affect any one of these individual reactions within the sequence to produce the same final response. Most drugs however, influence a number of stages in a variety of such sequences, resulting very often in a spectrum of apparently unrelated responses.

Discussion of Results

It is interesting to speculate upon the mechanism of the NAD-lowering action of these drugs. Either the synthesis or the destruction of NAD could be affected. It is certainly known that defects in the biosynthesis and inadequate dietary levels of the NAD precursor, nicotinamide, can provoke serious mental disturbances and deplete pyridine coenzymes, especially at the periphery. This explanation is however, unsatisfactory in the case of amphetamine and cocaine, since these drugs did not affect the rate of incorporation of ^{14}C-labelled nicotinamide into NAD in either the liver or brain. Nevertheless, there is a possibility that in an animal, treated with either of these drugs alone, the synthesis of NAD might well be affected. Such an effect could be obscured in this investigation by the presence of large quantities of nicotinamide, which might increase the rate of NAD synthesis, thereby offsetting the effects of amphetamine and cocaine.

Brain NAD levels might also be lowered as a result of either increased NADase activity or because

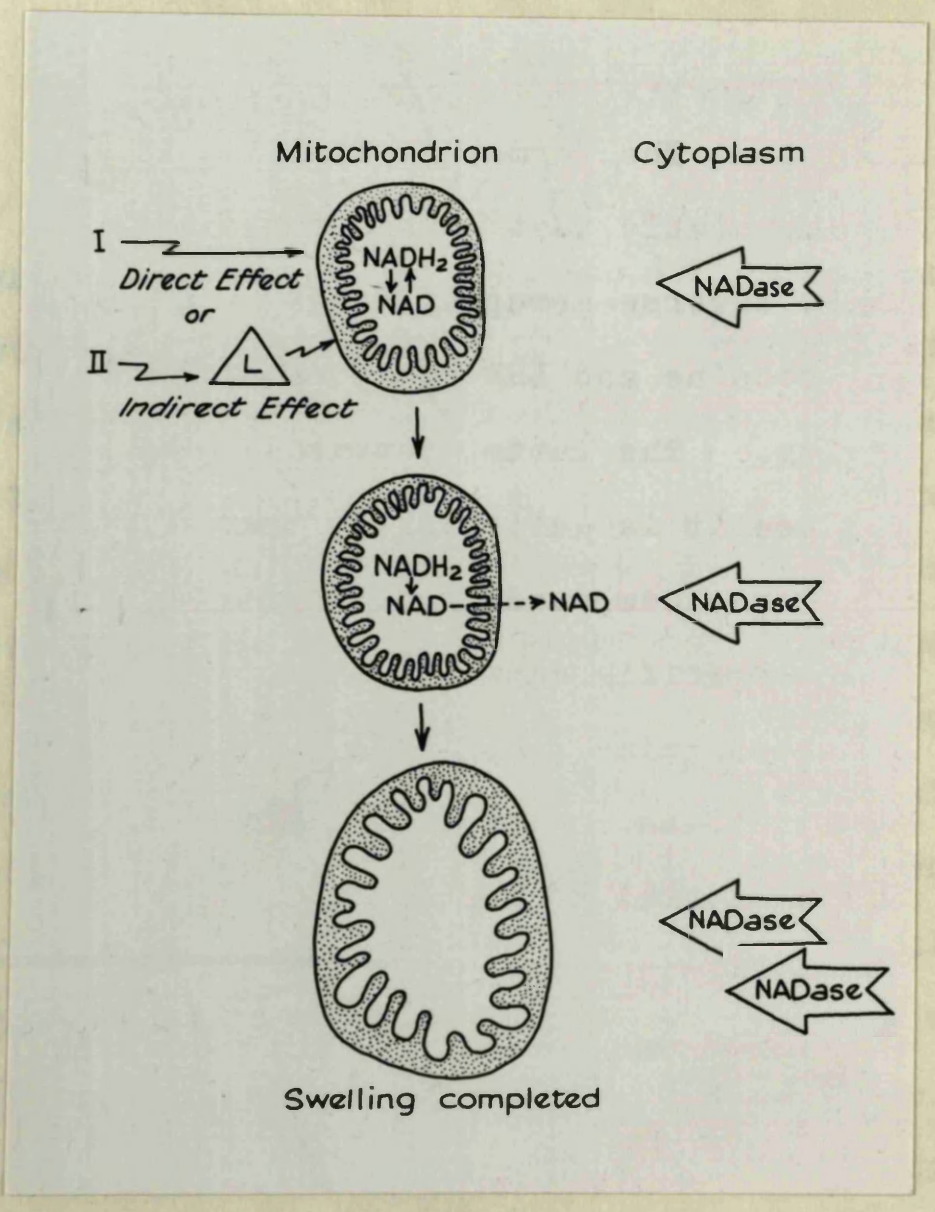
Mitochondrion
Cytoplasm
I
Direct Effect
or
II
L
Indirect Effect
NADH2
NAD
NADase
NADH2
NAD
NAD
NADase
NADase
NADase
Swelling completed

Fig. 72.

this enzyme encountered NAD normally unavailable for destruction. The former possibility is unlikely since it is improbable that such a pharmacologically and chemically diverse group of drugs as leptazol, amphetamine, cocaine and LSD could specifically increase NADase activity. The latter possibility is more attractive since it is quite likely that a variety of chemically diverse compounds could interfere in a relatively non-specific manner with the binding of NAD in distinct subcellular compartments, resulting in its destruction by NADase. This could occur if these drugs modified mitochondrial permeability, which can be affected by a variety of drugs (Ernster, Ikkos & Luft, 1959; Koch & Gallagher, 1960; Moore & Brody, 1960; Lucy, Luscombe & Dingle, 1963; Villela, 1964) and even by various physical treatments, including X-ray irradiation (MacCardle & Congdon, 1955). Such an effect might also be mediated indirectly through an initial action on the lysosomes (Fell, 1965).

In the process of mitochondrial swelling there is initially an interference with the enzymic reduction of NAD (Fig. 72), which then passes into the cytoplasm,

where it may be destroyed by the NADase, present mainly in the microsomes. These biochemical responses are accompanied by permeability changes in the mitochondrial membrane. This mechanism could be the basis of the *in vivo* NAD-lowering action of amphetamine and cocaine. However, the limited evidence, provided by this investigation, makes such a hypothesis unlikely, since drugs, which lowered brain NAD levels, merely produced *in vitro* aggregation of mitochondria and did not affect their water content.

Even this apparently unimportant response might be pharmacologically significant, especially since the dose required to produce it was low (5×10^{-5}M) and since the tranquilliser, chlorpromazine, produced the opposite effect at the same concentration. The aggregation reaction may be related to changes in adenine nucleotide metabolism (Mitchell & Sharp, 1964) and may depend upon drug-induced rearrangements of mitochondrial protein molecules (Joly, 1965). Supporting evidence is provided by histological studies, which show that chlorpromazine (Roizin, True & Knight, 1959) and LSD (Liss, Solze & Fischer, 1964) change the morphology

and staining properties of brain tissue. These physical effects may not represent the primary pharmacodynamic activities of amphetamine, cocaine, LSD and chlorpromazine but they may well point to them.

There is a very close physical and functional relationship between certain cerebral proteins and the pyridine coenzymes. The nicotinamide nucleotides may regulate the activity of a number of enzymes by altering the conformation of the enzyme protein (Grisolia, 1964). On the other hand, when cerebral protein metabolism is stimulated during chemically- or electrically-induced convulsions, which accelerate the turnover of amino acids, it is likely that concomitant changes could also occur in brain nicotinamide nucleotide metabolism. Such a violent disruption in cerebral functioning might be accompanied by the release and subsequent destruction of pyridine coenzymes together with the destruction of other constituents such as RNA (Talwar, Goel, Chopra & D'Monte, 1966). These changes need not necessarily occur only in the mitochondria. Nicotinamide nucleotides might also be released from their association with proteins in the

cytoplasm, which might be more readily affected by drugs than the mitochondria (Yates & Yates, 1964).

Such an effect would require only a very subtle modification in the structure or conformation of the NAD-protein complex (Colowick, van Eys & Park, 1966). These changes might be dependent upon the electronic properties of the drugs, which are in some cases very good electron donors (e.g. LSD) (Szent-Györgyi, 1960). It is also possible that the physical characteristics of a lattice (lipo-protein membrane) could be modified by the insertion of a drug molecule into the structure. For example, cocaine could become fixed in a membrane lattice in this way, causing the release of important ions or coenzymes (Mullins, 1954). Support for this hypothesis is provided by results, showing that X-ray irradiation lowered the threshold for electro-convulsive shock therapy, reduced brain catecholamines and sensitised mice to the lethal effects of amphetamine (Barnes, 1965). These actions could be caused by changes in the physical and functional integrity of some subcellular membranes (Szent-Györgyi, 1960).

On the other hand, chlorpromazine stabilises

membranes, reduces their permeability (Seeman & Bialy, 1963; Cooper, 1964) and decreases lysosomal activity (Lajtha & Toth, 1965). These actions may also be related to the electronic properties of chlorpromazine (Szent-Györgyi, 1960; Gallagher, Koch & Mann, 1965).

Drugs affecting brain NAD levels might exert their primary pharmacodynamic effects outside the central nervous system. For example, they might modify liver glycogen breakdown indirectly by releasing catecholamines. On the other hand, it is certainly possible that nicotinamide antagonises the NAD-lowering actions of cocaine and amphetamine by increasing blood glucose levels. This response may be mediated through the hypophysis (Greengard, Quinn & Reid, 1965). Some of the other drugs may also modify brain nicotinamide nucleotide metabolism indirectly, either through the thyroid (Moore, 1965) or the adrenals, which could in turn, affect the pituitary and the hypothalamus, where NADase activity is high.

In evaluating the present study it is necessary to compare the results with those of investigations on

closely related aspects of brain energy metabolism.

Attention has largely been focussed upon the actions of drugs on ATP, ADP and phosphocreatine levels and on the principal mechanisms which supply and require these substances within the brain. The relative concentrations of these "high energy" phosphates reflect the balance between the energy-requiring and energy-yielding reactions.

The "high-energy" phosphate content of the brain is reduced by anoxia, by drug or electrically-induced convulsions, by tranquillisers (Kaul et al, 1963a,b) and during all forms of hyperactivity. Brain ATP levels are increased by anaesthetics, amphetamine (Decsi, 1965), antidepressives (Lewis et al, 1963; Pecháň, 1965), psychotomimetics (Lewis et al, 1965) and hyperoxia (Decsi, 1965). It is therefore very difficult to associate specific changes in brain adenine nucleotide and phosphocreatine concentrations with particular levels of central nervous system activity. Drugs, which modify brain adenine nucleotides, seldom affect NAD (Pecháň, 1965), despite the very close functional association between the metabolism of these nucleotides

(Younathan, 1965), and the important regulating role of ATP in NAD synthesis (Nakamura, Nishizuka & Hayaishi, 1964). The characteristic stability of the nicotinamide nucleotides is also apparent in in vitro studies (McIlwain, Thomas & Bell, 1956; McIlwain, 1958a,b).

There is however, a marked difference in sensitivity between liver (Christie & Le Page, 1962a,b) and brain nicotinamide nucleotides to modification by drugs. For example, iproniazid reduces liver NAD levels but does not affect brain NAD (Bonasera, Mangione & Bonavita, 1963). Reserpine and chlorpromazine prolong the nicotinamide-elevated liver NAD levels more readily than those of brain (Burton, Kaplan, Goldin, Leitenberg & Humphreys, 1960; Burton, Salvador, Goldin & Humphreys, 1960).

Central depressants, including the barbiturates and tranquillisers, may interfere with substrate oxidation and oxygen uptake in isolated cerebral tissue (Quastel & Wheatley, 1932; Quastel, 1955; McIlwain & Greengard, 1957; McIlwain, 1959b; Wallgren & Kulonen, 1960; Cohen & Heald, 1960; Aldridge, 1962; Gey, Rutishauser &

Pletscher, 1965). They may also uncouple oxidative phosphorylation (Brody & Bain, 1954; Wolpert, Truitt, Bell & Krantz, 1956; Abood & Romanchek, 1957). However, the in vivo pharmacological significance of these observations is difficult to assess, since brain ATP and phosphocreatine levels do not fall during barbiturate anaesthesia but remain unchanged or even rise slightly (Schmidt, Kety & Pennes, 1945; Fazekas & Bessman, 1953; Lin, Cohen & Cohen, 1958).

The inhibition of glycolysis may be associated with decreased tissue NAD levels (Holzer, 1964). Supporting evidence is provided in this investigation by the NAD-lowering action of cocaine, which reduces oxygen uptake and glycolysis (Decsi, 1965). In contrast, amphetamine, which also reduced brain NAD levels in this study, stimulates glycolysis (Decsi, 1965).

Many questions remain unanswered. For example, it would be interesting to know whether the reduction in brain NAD levels corresponds in any way to that occurring in degenerating peripheral nerve tissue (Greengard, Brink & Colowick, 1954) and whether the fall occurs exclusively in one compartment (Kohen,

Siebert & Kohen, 1964) or generally throughout the cell.

The mechanism of action of nicotinamide in modifying the locomotor responses to amphetamine and cocaine requires further investigation. The main component in this action is probably the inhibitory effect upon NADase, which is associated with the toxicity of several drugs in the brain (Burton, 1960). However, nicotinamide possesses other properties, which might modify the response. Its ability to increase NAD synthesis, antagonise 5-HT, modify blood glucose levels and even abolish adrenochrome-induced abnormalities in the EEG (Szatmari, Hoffer & Schneider, 1955) may be relevant. In addition, nicotinamide increases tissue nucleic acid levels (Cima, Fassina & Pozza, 1960), an action which it shares with reserpine (Pevzner, 1966). The relative importance of these factors may vary with amphetamine and cocaine, resulting in different net effects.

Obviously too, the mechanisms of action of LSD and amphetamine are different. This is emphasised, despite their similar effects on NAD, by the disparity

between their locomotor responses and by the lack of cross-tolerance between them (Rosenberg, Wolbach, Miner & Isbell, 1963).

Yet, the interference with NAD metabolism cannot be entirely disregarded. Thus, succinate, which is mainly metabolised *via* the flavoproteins (Cristea, 1966) and therefore by-passes the nicotinamide nucleotides, blocks the psychotomimetic effects of LSD and mescaline (Gershon & Olariu, 1960). This suggests an interference at the nicotinamide nucleotide level. However, the interaction might not occur with the nucleotides themselves, but may be at some point in their synthesis or in a closely related biosynthetic pathway (Hersov & Rodnight, 1960), such as occurs in Hartnup disease.

This investigation does not permit any specific correlation to be made between psychotropic drug action and nicotinamide nucleotide metabolism. The small drug-induced reductions in brain nicotinamide nucleotide levels probably represent a general response, which may accompany central nervous system stimulation of diverse origin. Although such changes are unlikely to arise from a specific interference with a particular mechanism

(Fastier, 1964), as occurs with amylobarbitone and $NADH_2$ dehydrogenase (Giuditta, 1964), and probably do not explain the primary pharmacodynamic activities of these drugs, they may well become more significant during chronic administration (O'Hollaren, 1961). In these circumstances repeated changes in brain nicotinamide nucleotide levels, possibly associated with permanent alterations in the physical integrity of subcellular structures, may profoundly affect many of the major energy-producing mechanisms. Even then, it would be difficult to differentiate between these effects and the other direct and indirect actions of such drugs as amphetamine, LSD and cocaine on the metabolism of hypothetical central transmitters (van Rossum, 1963).

The results of the present investigation therefore permit only a very speculative and unsatisfactory correlation to be made between nicotinamide nucleotide metabolism and central nervous system drug action. Clarification of the importance of these effects demands a fuller understanding of the exact effects of drugs upon brain nicotinamide nucleotides and upon the related changes in structural organisation at the subcellular level.

Conclusions

Summary and Conclusions

The effects of a variety of centrally-acting drugs, including amphetamine and a number of related phenylethylamine derivatives, major tranquillisers, psychotomimetics, convulsants and other miscellaneous compounds upon rat brain nicotinamide nucleotide levels have been investigated. In addition, the effects of a number of these drugs upon locomotor activity, upon isolated brain mitochondrial swelling and upon the incorporation of radioactive nicotinamide into brain and liver NAD have been examined.

Throughout this study few significant changes in brain nicotinamide nucleotide levels were detected. Brain NAD levels were reduced by a chemically - diverse group of drugs, including amphetamine, cocaine, LSD, mescaline and leptazol. These drugs produced only very small changes in brain

NAD and did not cause any corresponding increase in the reduced nucleotide, suggesting a change in the total pyridine coenzyme content rather than an alteration in the dynamic equilibrium between the two forms. The changes in the brain NAD content produced by amphetamine and cocaine were not directly related to the locomotor stimulant actions of these drugs, since the time courses of the changes in the two parameters were not parallel. The dissociation of these two responses was further emphasised by LSD, which reduced brain NAD levels but did not modify the locomotor activity at all. Even in the cases of amphetamine and cocaine, which had apparently similar actions upon brain NAD levels and locomotor activity, there were important differences. Thus, nicotinamide inhibited the NAD-lowering responses to both drugs and reduced the behavioural effect of amphetamine but prolonged that of cocaine.

It is suggested that these drugs might

produce their NAD-lowering effects by releasing NAD either from the mitochondria or, as is more likely, from its close physical and functional association with enzyme protein in the cytoplasm. Such an action would then make the nucleotide available for destruction by NADase. This hypothesis is supported by evidence that the incorporation of radioactive nicotinamide into liver and brain NAD is unaffected by either amphetamine or cocaine. The disappearance of the cocaine and amphetamine-induced falls in brain NAD levels when the rats were treated simultaneously with nicotinamide may be explained on the basis of the inhibition of NADase by nicotinamide, which competes with the pyridine coenzymes for this and other enzymes (Murthy & Rappoport, 1963).

The in vitro studies showed that amphetamine and cocaine did not cause mitochondrial swelling. However, their ability to accelerate aggregation of mitochondria may at least reflect an ability to modify the physical properties of mitochondrial protein.

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Appendices

APPENDIX I

Sources and Quality of Reagents and Enzymes

Acetaldehyde: B.D.H., Reagent grade, ≥ 99.0%, acid ≤ 5 ml N/1 per 100 g. wt./ml = 0.778 - 0.781 g at 20°C.

Acetic acid: Hopkin & Williams, "Analar" glacial acetic acid, ≥ 99.6%.

Acetone: Hopkin & Williams, "Analar", ≥ 99%, distils between 56.0°C and 56.5°C.

Albumin: Sigma, bovine fraction V powder, 96 - 99%.

Alcohol dehydrogenase: Sigma, crystalline, from yeast, stock number 340-96, 90 mg protein dissolved in 0.5 ml of water, 1 mg protein reduces 165 µM of NAD/min at pH 8.8 and 25°C.

Ammonium acetate: B.D.H., "Analar".

Ammonium sulphate: B.D.H., "Analar", ≥ 99%.

Calcium chloride: B.D.H., "Analar", ≥ 98% (anhydrous).

APPENDIX I

Source and Quality of Reagents and Enzymes

Acetaldehyde: B.D.H., Reagent grade, ≮ 99.0%, acid ≯ 5 ml N/1 per 100 g, wt./ml = 0.778 - 0.781 g at 20°C.

Acetic acid: Hopkin & Williams, "Analar" glacial acetic acid, ≮ 99.6%.

Acetone: Hopkin & Williams, "Analar", ≮ 95%, distils between 56.0°C and 56.5°C.

Albumin: Sigma, bovine fraction V powder, 96 - 99%.

Alcohol dehydrogenase: Sigma, crystalline, from yeast, stock number 340-26, 50 mg protein dissolved in 0.5 ml of water, 1 mg protein reduces 165 µM of NAD/min at pH 8.8 and 25°C.

Ammonium acetate: B.D.H., "Analar".

Ammonium sulphate: B.D.H., "Analar", ≮ 99%.

Calcium chloride: B.D.H., "Analar", ≮ 98% (anhydrous).

Cytochrome c: Sigma, from horse heart, Type III, 90 - 100% based on molecular weight = 12, 270.

Dowex-1-chloride: Sigma, strongly basic anion, 4% cross linkage, 50 - 100 dry mesh, stock number 1 x 4 - 100.

EDTA: Sigma, ethylene diamine tetra acetic acid, disodium salt, 99.5%, stock number ED 2SS.

Ethanol: Burroughs, spectroscopic absolute alcohol.

Formic acid: B.D.H., Reagent Grade, 98 - 100%.

Glucose: Hopkin & Williams, "Analar".

Glucose-6-phosphate: Sigma.

Glucose-6-phosphate dehydrogenase: Sigma, Type V, from yeast. 1 unit reduces 1 μM of NADP/min at pH 7.4 and 25°C, 130 - 150 units/mg, 50 units dissolved in 0.5 ml of water.

Glycylglycine: B.D.H., Sigma.

Hydrochloric acid: Hopkin & Williams, "Analar" ≠ 35.4%, wt./ml = 1.18 g.

Hydrogen peroxide: Laporte, 30% H_2O_2 by weight.

Janus green B: T. & H. Smith.

Magnesium chloride: B.D.H., "Analar", ≮ 98%.

NAD: Sigma, β-nicotinamide adenine dinucleotide, Grade III, approximately 98%.

NADase: Sigma, β-NAD nucleotidase from N.crassa, 1 mg protein hydrolyses 4 μM of NAD in 7.5 min at pH 4.4 and 37°C, 5 mg dissolved in 2.0 ml of water.

$NADH_2$: Sigma, reduced β-nicotinamide adenine dinucleotide, Grade III, 98%.

$NADH_2$ cytochrome-c-reductase: Sigma, from pig heart, 1 mg reduces 0.5 μM of cytochrome c at pH 8.5 and 25°C, 100mg dissolved in 1.0 ml of water.

NADP: Sigma, nicotinamide adenine dinucleotide phosphate, sodium salt, 98 - 100%.

$NADPH_2$: Sigma, (chemically) reduced nicotinamide adenine dinucleotide phosphate, tetrasodium salt, Type I, 95 - 99%.

Nicotinamide: Sigma.

Nicotinamide (carbonyl - ^{14}C): Radiochemical centre, Amersham, 5 - 20 mc/mM.

Nitric acid: Hopkin & Williams, "Analar", 70% (within limits of 69.5 - 73.0%) wt./ml = 1.42.

NMN: Sigma, nicotinamide mononucleotide, 95 - 100%.

Potassium hydroxide: Hopkin & Williams, "Analar", $\leq$ 85%.

Potassium phosphate: B.D.H., Reagent Grade (K_2HPO_4).

Silver nitrate: B.D.H., N/1 volumetric solution.

Sodium chloride: B.D.H., "Analar", $\leq$ 99.9%.

Sodium dihydrogen phosphate: B.D.H., "Analar" (NaH_2PO_4 anhydrous).

Sodium hydrogen carbonate: Hopkin & Williams, "Analar", $\leq$ 99.5%.

Sodium hydrogen phosphate: B.D.H., "Analar" ($Na_2HPO_4.12H_2O$).

Sodium hydroxide: Hopkin & Williams, "Analar" $\leq$ 97%.

Sucrose: B.D.H., "Analar".

Trichloracetic acid: B.D.H., "Analar", $\leq$ 99%.

TRIS: Sigma, crystalline tris (hydroxymethyl) aminomethane, 99 - 99.5%.

APPENDIX II

APPENDIX II

Statistical Designs and Analyses

1. 3 x 3 Latin Square Design

In the first two series of experiments the rats were selected in groups of 3 of equal body weight. One rat in each group was injected with the control solution (C), while the other two were treated with different dose levels of the drug (L and H). The order of treatment and all subsequent procedures was randomised using three 3 x 3 latin squares.

As an example, the statistical design for series 2 and the analysis of variance of the NAD values in this experiment are shown in Table 1. In this series, the effects of chlorpromazine hydrochloride (15 mg/kg (L) and 30 mg/kg (H)) were investigated.

The results were then arranged in one table according to the treatment (Table 3) and in another (Table 4) in the same order in which they had been obtained. The means and standard errors of the means were calculated using the formulae shown in Table 2 for each set of variates.

The following functions were then calculated.

Table 2

Symbols Used in Calculation of the Standard Errors of the Mean

Symbol	Explanation
Σx	The sum of the individual values.
Σx^2	The sum of the squares of the individual values.
$(\Sigma x)^2$	The square of the sum of the individual values.
n	The number of observations.
$\bar{x}$	The mean = $\Sigma x / n$.
S.D.	The standard deviation from the mean = $\sqrt{\frac{\Sigma x^2 - (\Sigma x)^2 / n}{n - 1}}$
S.E.M.	The standard error of the mean = S.D. $/ \sqrt{n}$.

Table 1

3 x 3 Latin Square Design

Group	Row	Column 1	Column 2	Column 3
1	1	H	L	C
2	2	C	H	L
3	3	L	C	H
		Column 4	Column 5	Column 6
4	4	C	L	H
5	5	L	H	C
6	6	H	C	L
		Column 7	Column 8	Column 9
7	7	L	C	H
8	8	C	H	L
9	9	H	L	C

Table 2

Symbols Used in Calculation of the

Standard Errors of the Mean

Symbol	Explanation
Sx	The sum of the individual values.
Sx^2	The sum of the squares of the individual values.
$(Sx)^2$	The square of the sum of the individual values.
n	The number of observations.
$\bar{x}$	The mean = Sx/n.
S.D.	The standard deviation from the mean = $\sqrt{\frac{Sx^2 - (Sx)^2/n}{n - 1}}$
S.E.M.	The standard error of the mean = S.D./$\sqrt{n}$.

Table 3

Results Arranged According to Treatment

Group or Row	Control (C)	Chlorpromazine 30mg/kg (H)	Chlorpromazine 15mg/kg (L)
1	0.172	0.214	0.175
2	0.223	0.189	0.150
3	0.171	0.153	0.135
4	0.201	0.190	0.181
5	0.239	0.177	0.121
6	0.187	0.328	0.157
7	0.155	0.162	0.135
8	0.164	0.258	0.204
9	0.147	0.140	0.149
Symbol	**Value (C)**	**Value (H)**	**Value (L)**
Sx	1.659	1.811	1.407
Sx^2	0.313575	0.392347	0.225443
$(Sx)^2$	2.752281	3.279721	1.979649
$(Sx)^2/n$	0.305809	0.364413	0.219961
$\bar{x}$	0.184333	0.201222	0.156333
S.D.	0.031150	0.018690	0.026170
S.E.M.	0.010383	0.006230	0.008723

Table 4

Results Arranged According to Randomisation

Block 1	Column 1	Column 2	Column 3	Totals
Row 1	0.214	0.175	0.172	0.561
Row 2	0.223	0.189	0.150	0.562
Row 3	0.135	0.171	0.153	0.459
Totals	0.572	0.535	0.475	1.582
Block 2	**Column 4**	**Column 5**	**Column 6**	**Totals**
Row 4	0.201	0.181	0.190	0;572
Row 5	0.121	0.177	0.239	0.537
Row 6	0.328	0.187	0.157	0.672
Totals	0.650	0.545	0.586	1.781
Block 3	**Column 7**	**Column 8**	**Column 9**	**Totals**
Row 7	0.135	0.155	0.162	0.452
Row 8	0.164	0.258	0.204	0.626
Row 9	0.140	0.147	0.149	0.436
Totals	0.439	0.560	0.515	1.514

1. Correction Factor

= The square of the sum of all the observations, divided by the total number of observations.

$$= \frac{(1.582 + 1.781 + 1.514)^2}{27}$$

$$= 0.8809307$$

2. Total Sum of Squares

= The sum of the squares of all the observations minus the Correction Factor.

$$= (0.214)^2 + \ldots (0.140)^2 + (0.175)^2 + \ldots (0.147)^2 + (0.172)^2 + \ldots (0.149)^2 - \text{Correction Factor}.$$

$$= 0.931365 - 0.8809307$$

$$= 0.0504343$$

3. Treatment Sum of Squares

= The sum of the squares of the sum of all the observations for both dose levels of drug and for control, all divided by the number of observations, minus the Correction Factor.

$$= \frac{(1.659)^2 + (1.811)^2 + (1.407)^2}{9} - \text{Correction Factor}.$$

$$= 0.0092523$$

4. Blocks Sum of Squares

= The sum of the squares of the Totals for Blocks 1, 2 and 3, divided by the number of observations, minus the Correction Factor.

$$= \frac{(1.582)^2 + (1.781)^2 + (1.514)^2}{9} - \text{Correction Factor}$$

$$= 0.0042783$$

For the calculations which follow, the first term in the previous equation becomes the new Correction Factor. i.e. Block Correction Factor

$$= \frac{(1.582)^2 + (1.781)^2 + (1.514)^2}{9}$$

$$= 0.885209$$

5. Row Sum of Squares

= The sum of the squares of the Totals for Rows 1 to 9, divided by three, minus the Block Correction Factor.

$$= \frac{(0.561)^2 + (0.562)^2 + (0.459)^2 + (0.572)^2 + (0.537)^2 + (0.672)^2 + (0.452)^2 + (0.626)^2 + (0.436)^2}{3}$$

minus 0.885209

$$= 0.0130106$$

6. Columns Sum of Squares

= The sum of the squares of the Totals for Columns 1 to 9, divided by three, minus the Block Correction Factor.

$$= \frac{(0.572)^2 + (0.535)^2 + (0.475)^2 + (0.650)^2 + (0.545)^2 + (0.586)^2 + (0.439)^2 + (0.560)^2 + (0.515)^2}{3}$$

minus 0.885209

= 0.0059580

7. Error Sum of Squares

= The difference between the Total Sum of Squares and the sum of the Treatment Sum of Squares, the Block Sum of Squares, the Row Sum of Squares and the Column Sum of Squares.

= 0.0504343 - (0.0092523 + 0.0042783 + 0.0130106 + 0.005958)

= 0.0179351

Table 5

Analysis of Variance

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F
Blocks	2	0.0042783	0.00213915	1.19
Rows	6	0.0130106	0.0021684	1.21
Columns	6	0.0059580	0.0009930	0.55
Treatment	2	0.0092523	0.0046261	2.58
Error	10	0.0179351	0.0179351	
Total	26	0.0504343		

Analysis of Variance

In order to facilitate the calculation of the F values, the results obtained in the foregoing calculations were tabulated (Table 5).

Significance: Since the F value for Treatment was less than $F_{0.05,}$ there was no significant difference between the drug and control values.

2. Simple Randomisation of Two Treatments

The rats were selected in pairs of equal body weight. The order in which the animals were injected, killed and their brains dissected, extracted and assayed was randomised using a table of random numbers. Equal chances were given for the control and drug treated animal in each pair to come first or second by deciding arbitrarily that a number between 0 and 24 indicated that the control was to come first, while a number between 25 and 49 indicated that the drug was to come first. An example of an experimental design obtained in this way is given in Table 6. This design was used in the investigation of the effects of d-amphetamine sulphate (10 mg/kg). The calculations of the means and standard errors of the means and the analysis of variance for the NAD results are given below.

The data were arranged in a table according to treatment (Table 7). The following functions were then calculated.

1. Correction Factor

$$= \frac{(1.980599 + 1.628420)^2}{20}$$

$$= 0.65125$$

Table 6

Randomisation of Two Treatments

Group Number	Order of Treatment	
	First	Second
1	Drug	Control
2	Control	Drug
3	Drug	Control
4	Drug	Control
5	Control	Drug
6	Drug	Control
7	Control	Drug
8	Control	Drug
9	Drug	Control
10	Control	Drug

Table 7

Results Arranged According to Treatment

Group	Control (C)	Drug (D)	C + D
1	0.163551	0.138053	0.301604
2	0.186403	0.144824	0.331227
3	0.190075	0.193004	0.383079
4	0.194530	0.165110	0.359640
5	0.199167	0.185681	0.384848
6	0.203324	0.105146	0.308470
7	0.200278	0.175031	0.375309
8	0.223127	0.163406	0.386533
9	0.210739	0.219582	0.430321
10	0.209405	0.138583	0.347988

Symbol	Value (C)	Value (D)	Value (C + D)
Sx	1.980599	1.628420	3.609019
Sx^2	0.39463189	0.2748365	1.316565
$(Sx)^2$	3.922772399	2.6517516964	
$(Sx)^2/n$	0.3922772399	0.26517516964	
S.D.	0.016175	0.010361	
S.E.M.	0.00511498	0.00327643	
$\bar{x}$	0.1980599	0.162842	

Table 8

Analysis of Variance

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F
Groups	9	0.0070325	0.000781	1.4086
Drug Versus Control	1	0.0062024	0.00062024	11.18
Error	9	0.00499	0.0005544	
Total	19	0.01822		

2. Total Sum of Squares

$= (0.163551)^2 + (0.186403)^2 + \ldots (0.209405)^2 +$
$(0.138053)^2 + \ldots (0.138583)^2 - 0.651250$

$= 0.66946839 - 0.651250$

$= \underline{0.01821839}$

3. Drug Versus Control Sum of Squares

$$= \frac{(1.980599)^2}{10} + \frac{(1.628420)^2}{10} - 0.651250$$

$= 0.6574524095 - 0.651250$

$= \underline{0.0062024095}$

4. Between Groups Sum of Squares

$$= \frac{(0.301604)^2}{2} + \frac{(0.331227)^2}{2} + \ldots \frac{(0.347988)^2}{2} - 0.651250$$

$= 0.6582825 - 0.651250$

$= \underline{0.0070325}$

5. Error Sum of Squares

$= 0.01821839 - (0.0062024095 + 0.0070325)$

$= \underline{0.00499}$

In order to facilitate the calculation of the F values, the results obtained in the foregoing calculations were again tabulated (Table 8).

Significance: Since the F value for the Drug Versus Control (Treatment) was greater than $F_{0.01}$, there was a significant difference between the drug and control values. i.e. F lay between $F_{0.01}$ and $F_{0.001}$.

APPENDIX III

Preparation of ALGOL Programme for the Analysis of Variance in an Experiment Involving Two Treatments

The programme was written in KDF 9 ALGOL (Backus, Bauer, Green, Katz, McCarthy, Naur, Perlis, Rutishauser, Samelson, Vauquois, Wegstein, Van Wijngaarden, Woodger, 1963; Green, 1963). In order to facilitate the interpretation of this programme, the symbols (identifiers) were chosen to correspond as closely as possible to conventional statistical nomenclature and to indicate the meaning of each function.

The basic equations required for calculating the means, the standard errors of the means and the analysis of variance were derived using the identifiers shown in Tables 9 and 10.

The programme was then written as follows.

```
begin comment        This is a programme for the
      calculation of the means and standard errors
      of the means and for the analysis of variance
      in an experiment in which two groups of animals
      were treated with drug or control solution ;
```

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```
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      calculation of the means and standard errors
      of the means and for the analysis of variance
      in an experiment in which two groups of animals
      were treated with drug or control solution ;
```

Table 9

ALGOL Identifiers Used in Programme

Drug Identifiers	Drug + Control Identifiers	Control Identifiers	Explanation
Sx	Sxy	Sy	Sum of values
Ssqx	Ssqxy	Ssqy	Sum of $(\text{values})^2$
sqSx	sqSxy	sqSy	$(\text{Sum of values})^2$
AvSx		AvSy	Sum of values/Number of observations
AvsqSx		AvsqSy	$(\text{Sum of values})^2$/Total number of observations
sdx		sdy	Standard deviation
semx		semy	Standard error of mean
drugRF		controlRF	Relative fluorescence values
DblankRF		CblankRF	Relative fluorescence values for blank
TRUEdrugRF		TRUEcontrolRF	Corrected relative fluorescence values
TOTALdrug		TOTALcontrol	Values expressed as micromoles
Dbrain weight		Cbrain weight	Brain weights
DRUG	SUM	CONTROL	Values expressed as micromoles/g

```
integer i, j, m, n, fa, fb, fc, fd, fe ;
real Sx, Sy, Sxy, Ssqx, Ssqy, Ssqxy, sqSx,
sqSy, sqSxy, AvsqSx, AvsqSy, AvSx, AvSy,
sdx, sdy, semx, semy, CF, TSS, DVCSS, cfd,
cfc, BGSS, ESS ;

fa := format ( [ss-ndsssssssssss] ) ;
fb := format ( [ss-nd.ddddddssssss] ) ;
fc := format ( [-nd.dddddddsssss] ) ;
fd := format ( [s-nd.ddddddds] ) ;
fe := format ( [ss-nd.ddddddds] ) ;

open (20) ;
m := read (20) ;
for j := 1 step 1 until m do
begin n := read (20) ;
    cfd := read (20) ;  cfc := read (20) ;
    Sx := Sy := Sxy := Ssqx := Ssqy := Ssqxy := 0.0 ;
    begin  array  drugRF, controlRF, DblankRF, CblankRF,
          TRUEdrugRF, TRUEcontrolRF, TOTALdrug, TOTALcontrol,
          Dbrain weight, Cbrain weight, DRUG, CONTROL, SUM[1 :n];
          for i := 1 step 1 until n do
```

Table 10

ALGOL Identifiers Used in Programme

Function	Identifier	Explanation
Correction Factor	CF	sqSxy/(n+n)
Total Sum of Squares	TSS	Ssqx + Ssqy - CF
Drug versus Control Sum of Squares	DVCSS	AvsqSx + AvsqSy - CF
Between groups Sum of Squares	BGSS	(Ssqxy/2.0) - CF
Error Sum of Squares	ESS	TSS - DVCSS - BGSS
Calibration Factor (Drug)	cfd	Converts relative fluorescence values to micromoles
Calibration Factor (Control)	cfc	

```
begin drugRF [i] := read (20) ;
      controlRF [i] := read (20) ;
      DblankRF [i] := read (20) ;
      CblankRF [i] := read (20) ;
      Dbrain weight [i] := read (20) ;
      Cbrain weight [i] := read (20) ;
      if DblankRF [i] < drugRF [i] then
      TRUEdrugRF [i] := drugRF [i] - DblankRF [ i ]
      else TRUEdrugRF [i] := 0.0 ;
      if TRUEdrugRF [i] > 0.0 then
      TOTALdrug [i] := TRUEdrugRF [i] X cfd
      else TOTALdrug [i] := 0.0 ;
      if TOTALdrug [i ] > 0.0 then
      DRUG [ i ] := TOTALdrug [i ] / Dbrain weight [ i ]
      else DRUG [i ] := 0.0 ;
      if CblankRF [i ] < controlRF [i ] then
      TRUEcontrolRF [i ] := controlRF [i ] -CblankRF [ i ]
      else TRUEcontrolRF [i ] := 0.0 ;
      if TRUEcontrolRF [i ] > 0.0 then
      TOTALcontrol [i] := TRUEcontrolRF [i ] X cfc
      else TOTALcontrol [i ] := 0.0 ;
      if TOTALcontrol [i ] > 0.0 then
```

```
    CONTROL [i ] := TOTALcontrol [i ] / Cbrain weight [i ]
    else CONTROL [i ] := 0.0 ;
    SUM [i ] := DRUG [i ] + CONTROL [i];
    Sx := Sx + DRUG [i ];
    Sy := Sy + CONTROL [i ];
    Sxy := Sxy + SUM [i ] ;
    Ssqx := Ssqx + DRUG [i ] X DRUG [i ] ;
    Ssqy := Ssqy + CONTROL [i ] X CONTROL [i ] ;
    Ssqxy := Ssqxy + SUM [i ] X SUM [i ]
end;
sqSx := Sx X Sx ;
sqSy := Sy X Sy ;
sqSxy := Sxy X Sxy ;
AvsqSx := sqSx/n ;
AvsqSy := sqSy/n ;
AvSx := Sx/n ;
AvSy := Sy/n ;
sdx := sqrt (( Ssqx - AvsqSx )/(n-1)) ;
semx := sdx/sqrt (n) ;
sdy := sqrt ((Ssqy - AvsqSy)/(n-1)) ;
semy := sdy/sqrt (n) ;
CF := sqSxy/(n + n) ;
TSS := Ssqx + Ssqy - CF ;
```

```
DVCSS := AvsqSx + AvsqSy - CF ;
BGSS := (Ssqxy/2.0) - CF ;
ESS := TSS - DVCSS - BGSS ;
open (10) ;
write text (10, [Results * for * means * and *
standard * errors * of * means  [5c5s]
Mean * for * drug * = *] ) ;
write (10, fb, (Sx/n)) ;
write text (10, [[2c5s] Standard * error*
of * mean * for * drug * = *] ) ;
write (10, fb, semx) ;
write text (10 [[5c5s] Mean * for *
control * = *]) ;
write (10, fb, (Sy/n)) ;
write text (10, [[2c5s] Standard * error *
of * mean * for * control * = *]) ;
write (10, fb, semy ) ;
write text (10, [[6c] Variation * due * to] ) ;
write text (10, [[4s] Degrees * of * freedom] ) ;
write text (10, [[4s] Sum * of * squares] ) ;
write text (10, [[4s] Mean * square] ) ;
write text (10, [[10s] F ] ) ;
write text (10, [[3c6s] Treatment [11s]] ) ;
```

```
write (10, fa, 1 ) ;
write (10, fb, DVCSS ) ;
write (10, fc, DVCSS ) ;
write (10, fd, (DVCSS X (n - 1)/ ESS )) ;
write text (10, [ [2c6s ] Rows [16s]] ) ;
write (10, fa, (n - 1)) ;
write (10, fb, BGSS ) ;
write (10, fc, (BGSS/(n - 1))) ;
write (10, fd, (BGSS/(n - 1))/(ESS/(n - 1))) ;
write text (10, [ [2c6s ] Error [15s ] ] ) ;
write (10, fa, (n - 1)) ;
write (10, fb, ESS) ;
write (10, fc, ESS/(n - 1)) ;
write text (10, [ [2c6s ]  Total [15s]]) ;
write (10, fa, ((n + n)-1)) ;
write (10, fb, TSS) ;
write text (10, [ [3c ] Standard * error * of *
a * single * observation ** = * ] ) ;
write (10, fe, sqrt (ESS/(n - 1))) ;
write text (10, [ [2c ] Grand * mean [18s ] = ] ) ;
write (10, fe, ((Sx + Sy)/(n * n ))) ;
write text (10, [ [2c ] Coefficient * of *
variation **** = ] ) ;
```

```
write (10, fe, (sqrt (ESS/(n - 1))/((Sx + Sy)/(n + n)))) ;
write text (10, [[10c ]] ) ;
close (10)
end array block
end for loop ;
close (20)
end
```

→

Table 11

Layout of Data Prepared for the Computer

```
1;
10.;
0.07006;
0.07006;
7.45;  6.80;  1.55;  1.50;  1.51;  1.58;
6.20;  6.50;  1.55;  1.40;  1.45;  1.60;
6.10;  6.00;  1.50;  1.55;  1.44;  1.41;
6.55;  5.75;  1.50;  1.40;  1.45;  1.51;
5.90;  5.25;  1.45;  1.60;  1.43;  1.49;
6.95;  5.70;  1.55;  1.40;  1.46;  1.48;
5.80;  5.20;  1.40;  1.20;  1.48;  1.43;
5.45;  6.40;  1.25;  1.50;  1.40;  1.54;
7.30;  3.50;  1.65;  1.05;  1.40;  1.16;
6.10;  3.70;  1.50;  1.15;  1.53;  1.15;
```

→

Table 12

Layout of Results obtained from the Computer

Results for means and standard errors of means

Mean for drug = 0.235522

Standard error of mean for drug = 0.008746

Mean for control = 0.197866

Standard error of mean for control = 0.009564

Variation due to	Degrees of Freedom	Sum of squares	Mean square	F
Treatment	1	0.007090	0.007090	8.034933
Rows	9	0.007176	0.000797	0.903574
Error	9	0.007941	0.000882	
Total	19	0.022207		

Standard error of a single observation = 0.029705

Grand mean = 0.216694

Coefficient of Variation = 0.137082

The experimental data were presented to the computer in the form shown in Table 11. The results were provided by the computer in the form shown in Table 12. The data used in this example were those obtained for NAD in the investigation using nicotinamide (250 mg/kg).

Both the programme and the data were transferred to 8-channel paper tape and were used in this form in the computer. The results, which were also "output" in this form, were then printed on an off-line flexowriter.

Preparation of ALGOL Programme for a Simple t-Test

This programme was written in KDF 9 ALGOL using many of the symbols (identifiers), previously described for the variance analysis programme (Table 9). In addition, the standard deviation from the mean for two sets of variates was represented by the symbol SDxy. The "t" value was represented by the symbol T.

The programme was then written as follows.

```
begin comment  This programme calculates the
      means and standard errors for drug and control values
      and carries out a t test on the results;
      integer i, j, k, m, nx, ny, fa;
      real Sx, Sy, Ssqx, Ssqy, sqSx, sqSy,
      AvsqSx, AvsqSy, AvSx, AvSy, sdx, sdy, semx,
      semy, SDxy, T;
      fa:= format ( [ ss - nddd.dddss ] );
      open (20);
      open (10);
      m:= read (20);
      for i:= 1  step 1 until m do
      begin nx:= read (20);
            ny:= read (20);
            Sx:= Sy:= Ssqx:= Ssqy:= 0.0;
            begin array DRUG [1:nx], CONTROL [1:ny];
                  for j:= 1 step 1 until nx do
                  begin DRUG [j]:=  read (20);
                        Sx:= Sx+DRUG [j];
                        Ssqx:= Ssqx+DRUG [j]↑2;
                    end;
                  for k:= 1 step 1 until ny do
                  begin CONTROL [k]:= read  (20);
```

```
            Sy:= Sy+CONTROL [k];
            Ssqy:= Ssqy + CONTROL[k]↑2;
        end;
    end;
sqSx:= Sx ↑2;
sqSy:= Sy ↑2;
AvsqSx:= sqSx/nx;
AvsqSy:= sqSy/ny;
AvSx:= Sx/nx;
AvSy:= Sy/ny;
sdx:= sqrt ( (Ssqx - AvsqSx)/(nx - 1) );
sdy:= sqrt ( (Ssqy - AvsqSy)/(ny - 1) );
semx:= sdx/sqrt ( nx );
semy:= sdy/sqrt ( ny );
SDxy:= sqrt (sdx↑2/nx + sdy↑2/ny);
if AvSx> AvSy then T:= (AvSx - AvSy)/SDxy else
T:= (AvSy - AvSx)/SDxy;
write text (10, [ [5c] Mean*f or *drug* = * ]);
write (10, fa, AvSx);
write text (10, [ [2c ] Standard *error* of *mean*
for *drug* = * ] );
write (10, fa, semx);
write text (10, [ [3c] Mean* for *control* = * ]);
```

```
            write (10, fa, AvSy);
            write text (10, [[2c] Standard *error* of *mean*
            for *control* = *]);
            write (10, fa, semy);
            write text (10, [[3c] Result *of *t *test* =*]);
            write (10, fa, T);
            end;
   close (10);
close (20);
end; ⟶
```

Table 13

Layout of Data Prepared for the Computer

```
1;
4;
4;
 90.0;   85.5;   84.5;   92.0;
118.0;   88.0;   86.5;   73.0;
```

→

Table 14

Layout of Results Obtained from the Computer

Mean for drug = 88.000

Standard error of mean for drug = 1.791

Mean for control = 91.375

Standard error of mean for control = 9.494

Result of t test = 0.349

The experimental data were presented to the computer in the form shown in Table 13. The results were provided by the computer in the form shown in Table 14.

In this case the programme and data were transferred to 8-channel paper tape, the final results again being printed out on an off-line flexowriter.

APPENDIX IV

Preparation of Enzymes

1. NADP - "Cytochrome c Reductase"

A variety of yeasts including bottom lager, bottom ale and a mixed yeast were used as starting materials. In this stage the enzyme was liberated by autolysis. Approximately 5 kg of moist yeast, obtained from a filter press, was suspended in 14 l of water, initial mixing being carried out in a Waring blender. This suspension was incubated for 33 hr at 20°C in a thermostatically-controlled room. During this time the yeast was occasionally stirred to promote autolysis (Fig. 26).

After centrifuging the suspension (500xg, 30 min, 20°C), the supernatant was set aside. The precipitate was re-extracted by suspending in water (6 l) and centrifuging (500xg, 30 min, 20°C). The combined supernatants were then cooled to 0°C and ammonium sulphate added with continuous stirring to produce a saturation of 51%. The pH of the solution was adjusted to 4.5 using acetic acid (10 N) and the resulting

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precipitate, containing the enzyme, was separated by centrifugation (500 x g, 30 min, 0°C). This material was maintained at 0°C until required for the next stage, in which the inactive protein contaminants were removed by ammonium sulphate fractionation.

The precipitate was dissolved in water (700 ml, 2°C), to which was added 3 l of a 31% saturated solution of ammonium sulphate (0°C). After separating the precipitate by centrifuging (1000 x g, 30 min, 0°C), the concentration of ammonium sulphate in the supernatant was increased to 51%. The resulting precipitate was removed by centrifuging (1000 x g, 30 min, 0°C) and was dissolved in water (500 ml, 2°C). This solution was dialysed against running water for 17 hr at 5°C. The precipitate, which occasionally formed during dialysis, was removed by centrifuging (1000 x g, 30 min, 0°C). The pH of the supernatant was adjusted to 4.65 by adding potassium hydroxide (2 N). Ethanol (600 ml, 30% v/v, 0°C) was added very slowly to the supernatant and the resulting fine precipitate, which generally formed after 45 min was removed by centrifuging (3000 x g, 45 min, 0°C) and was dissolved in water (20 ml, 2°C).

This solution was frozen as a thin layer on the inner surface of a round bottomed flask, rotated rapidly in a freezing mixture (acetone-solid carbon dioxide). The frozen enzyme solution was freeze-dried using a Quickfit lyophiliser (Assembly 1 LT) and a two stage rotary vacuum pump (Leybold-Elliott, model 62, Type D2). The powder containing the enzyme was finally stored at $-15^{\circ}C$.

Assay of NADP - "Cytochrome c Reductase". The powdered enzyme was dissolved in an albumin (1% w/v) - glycylglycine (0.01 M) buffer solution (5 ml, pH 7.6, $2^{\circ}C$) and was assayed using an adaptation of the method employed by Mahler, Sarkar, Vernon and Alberty (1952) for standardising NAD- "cytochrome c reductase". Aliquots of this solution were transferred to a 1 cm cell containing

(1) cytochrome c (0.105 µM)

(2) $NADPH_2$ (0.60 µM)

(3) TRIS buffer (0.67 µM, pH 8.5)

(4) water to 3.0 ml.

The activity of the enzyme was measured by following the rate of reduction of cytochrome c at 550 mµ using a Hilger-Uvispek spectrophotometer. Optical density

readings were recorded at 15 sec intervals for 10 min and the enzymic activity expressed as units/ml, one unit being defined as the amount required to produce an optical density rise of 1.0/min (Mahler *et al*, 1952).

2. Glucose-6-Phosphate Dehydrogenase

Approximately 100 g of moist bottom lager yeast, obtained from a filter press, was suspended in sodium bicarbonate solution (300 ml, 0.1 M, saturated with carbon dioxide) and maintained at 40°C for 5 hr (Fig. 31). The autolysed suspension was centrifuged (3000 x g, 30 min, 2°C) and sodium bicarbonate solution (530 ml, 0.1 M, 2°C, saturated with carbon dioxide) and ammonium sulphate (250 g) were then added slowly to the supernatant with constant stirring. After centrifuging (3000 x g, 30 min, 2°C), the precipitate was discarded and ammonium sulphate (53 g) was added slowly to the supernatant. The resulting precipitate was removed by centrifuging (5000 x g, 30 min, 2°C) and was dissolved in water (1000 ml, 2°C). This solution was passed through a chromatography column (0.5 ml/min), containing 300 ml of calcium phosphate gel, prepared by the interaction of dilute aqueous solutions of calcium chloride and sodium phosphate (Keilin & Hartree, 1938). The adsorbed enzyme was eluted with 450 ml of phosphate buffer (0.5 M, pH 7.7, 2°C). Ammonium sulphate (150 g) was then added to the eluate and the resulting precipitate

was separated by centrifuging (5000 x g, 30 min, 2°C) and discarded. A second precipitate, obtained by adding 26 g of ammonium sulphate to the supernatant, was separated by centrifuging (8000 x g, 45 min, 2°C) and was dissolved in water (90 ml, 2°C). Acetate buffer (450 ml, 0.1 M, pH 4.4, 2°C) was added to the supernatant, which was then cooled to 0°C for 15 min. The fine precipitate, which formed during this period, was removed by centrifuging (8000 x g, 30 min, 0°C), dissolved in glycylglycine buffer (18 ml, 0.25 M, pH 7.4, 2°C) and freeze-dried using a Quickfit lyophiliser and two-stage rotary vacuum pump. The powder containing the enzyme was finally stored at -15°C.

Assay of Glucose-6-Phosphate Dehydrogenase. The enzyme activity was measured spectrophotometrically by following the increase in optical density at 340 mμ during the reduction of NADP. The powdered enzyme preparation was dissolved in glycylglycine buffer (0.25 M, pH 7.4, 2°C) and an aliquot of this solution was transferred to a 1 cm cell containing

(1) 0.5 ml of glycylglycine buffer
(0.25 M, pH 7.4, 2°C)

(2) 0.1 ml of magnesium chloride (0.3 M)

(3) 0.1 of NADP (0.0015 M)

(4) 0.1 ml of glucose-6-phosphate (0.025 M)

(5) water to 3.0 ml.

Optical density readings were recorded at regular intervals for 2 min. A unit of glucose-6-phosphate dehydrogenase activity was defined as the amount of enzyme required to produce an optical density change of 2.07/min (Kornberg, 1950).

APPENDIX V

Preparation of the Dowex-1-Formate Chromatography Column

Conversion of the Resin Chloride to Formate

Approximately 200 g of Dowex-1-chloride (4% cross linked, dry mesh 50-100) was washed with running water (~ 10 l, 15 min). Occasional stirring and decanting facilitated the removal of soluble impurities and the very fine resin particles. The resin was treated with sodium hydroxide (4 N, 3 l, 15 min) and then washed again with running water (~ 10 l, 15 min). After decanting the water, the resin was converted to Dowex-1-formate by adding formic acid (4 N, 3 l, 1 hr). A further period of washing (~ 10 l water, 15 min) removed all chloride ions. This was confirmed by testing the decanted water for chloride using silver nitrate (N) and nitric acid (N).

Preparation of the Column

A chromatography column consisting of a glass tube 54 cm long and having an internal diameter of 0.7 cm, was used to separate brain NAD (Berg, 1963). A small glass wool plug approximately 2 cm long was inserted at

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one end of the tube, to which was also attached polythene tubing (12 cm). The opposite end of the column was immersed in a slurry of resin and water. Suction applied to the polythene tubing filled the column with resin. The polythene tubing was then clamped with artery forceps, and the packed height of the inverted column adjusted to 50 cm by adding or removing resin slurry. A glass wool plug was also inserted at the open end and polythene tubing (6 cm) attached to connect the column to a filter funnel reservoir. This method of filling the tube produced a homogeneous resin bed free from air bubbles. The flow rate through the column was finally regulated to 0.25 ml/min using a screw clip.

Abstract

STUDIES ON THE MODE OF ACTION OF CENTRALLY-ACTING DRUGS

Abstract of a thesis submitted to the
University of Glasgow
in candidature for the degree of
Doctor of Philosophy
in the
Faculty of Science

by

David Pollock, M.Sc.

Division of Experimental Pharmacology,
Institute of Physiology,
The University,
Glasgow.

April, 1967.

This thesis is primarily concerned with the effects of a number of tranquillisers, central stimulants, antidepressives and psychotomimetics upon rat brain nicotinamide nucleotide metabolism. Certain other related aspects of the activities of these drugs, including their effects upon locomotor activity and mitochondrial swelling, are also examined.

In the introduction the urgent need for understanding the basic mechanisms of action of psychotropic drugs is emphasised and possible experimental approaches to this problem are considered. A survey of the part played by pyridine coenzymes in the main pathways of energy metabolism is given. Justification for the present investigation is mainly provided by certain pharmacological and clinical observations, suggesting possible connections between nicotinamide nucleotide metabolism, altered mental performance and psychotropic drug action. This evidence is reviewed.

Spectrophotometric and spectrophotofluorometric assays for brain nicotinamide nucleotides are described and evaluated. The isolation of the necessary accessory

enzyme is described in detail. Methods of measuring the effects of drugs on *in vitro* mitochondrial swelling, on the *in vivo* incorporation of radioactive nicotinamide into NAD and upon locomotor activity are outlined. Two ALGOL programmes, devised to permit the statistical analysis of data using a digital computer, are described.

A number of central nervous system stimulants and psychotomimetics reduced brain NAD levels without affecting the $NADH_2$ levels. The fall in brain NAD levels therefore indicated a change in the total nucleotide content rather than an alteration in the dynamic equilibrium between the oxidised and reduced forms. Tranquillisers had no direct effect upon brain nicotinamide nucleotide levels. Certain central stimulants, which lowered brain NAD levels also apparently accelerated spectrophotometrically-determined mitochondrial swelling. The discrepancy between this latter result and the lack of effect observed in gravimetric measurements of mitochondrial swelling was resolved by microscopy, which revealed that these drugs caused *in vitro* aggregation. Attempts were made to

correlate the drug-induced reduction in brain NAD levels with alterations in the rate of incorporation of radioactive nicotinamide into liver and brain NAD. Behavioural studies showed that the locomotor stimulant effects produced by some of these drugs could be modified by the simultaneous administration of nicotinamide, which also increased brain NAD levels. The time course of the behavioural-stimulant effect did not however, parallel the NAD-lowering action.

In the discussion the experimental methods are critically assessed and the validity and pharmacological relevance of the results discussed with reference to current theories of psychotropic drug action. The particular significance of these results is suggested to lie not in the amount by which brain NAD levels fell but in the fact that any reduction occurred at all, since brain nicotinamide nucleotides are notably unaffected even by procedures, which produce very severe depletion of NAD at the periphery. An examination is made of the various mechanisms by which brain NAD levels could be lowered. The possibility that drug-induced falls in brain NAD levels could arise from a relatively non-specific

physical effect upon subcellular structures is examined. The implications of this hypothesis are reviewed and the need for further research to determine the exact nature of the relationship between structure and function at the subcellular level is indicated.

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SHORT COMMUNICATIONS

Effects of *d*-Amphetamine and Chlorpromazine on Oxidised (NAD) and Reduced ($NADH_2$) Nicotinamide Adenine Dinucleotide Levels in Rat Brain

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LARGE doses of nicotinamide increase liver, brain and spleen NAD.[1, 2] Nicotinic acid has a similar effect on blood NAD.[3, 4] Reserpine or chlorpromazine, given prior to nicotinamide, maintain elevated liver NAD levels.[5] The ability of brain tissue to control NAD metabolism is reflected by the absence of this reserpine effect in brain and because nicotinamide itself causes only a 50–75% increase in brain NAD[2] but an 800–900% increase in the liver.[1] Furthermore, when peripheral NAD stores are severely depleted by dietary deficiences,[6] no change in brain NAD occurs, even in terminally deficient animals.[2]

We have investigated the effects of *d*-amphetamine sulphate and chlorpromazine hydrochloride on rat brain NAD and $NADH_2$ levels to determine if the influence of these drugs on behaviour could be correlated with changes in NAD metabolism.

Drug solutions were prepared in 0·9% NaCl so that the required dose was contained in 0·2 ml/100 g body weight. Groups of two or three male Wistar rats of approximately equal weight (90–120 g) were used. The order in which the animals were injected intraperitoneally, killed and the brains dissected, extracted and assayed, was randomised, using either a 3 × 3 Latin Square design or a table of random numbers. 0·9% NaCl was used as control. The method for extracting and estimating the nicotinamide

nucleotides was essentially that of Lowry *et al.*,[7] using the whole brain instead of a sample. The experimental data were evaluated by means of the appropriate variance analysis.

The results are shown in Table 1. Only *d*-amphetamine (10 mg/kg) produced a statistically significant fall in the NAD content. No significant changes were observed in the $NADH_2$ levels.

TABLE 1. *In vivo* EFFECTS OF *d*-AMPHETAMINE AND CHLORPROMAZINE ON RAT BRAIN NAD AND $NADH_2$ LEVELS

Series	Treatment	Dose (mg/kg)	Time after treatment (hr)	Concentration (μmoles/g) NAD	$NADH_2$
1	Control		3	0·198 ± 0·011	0·020 ± 0·003
	d-Amphetamine	5		0·170 ± 0·012	0·021 ± 0·004
	d-Amphetamine	2·5		0·164 ± 0·010	0·020 ± 0·003
2	Control		3	0·184 ± 0·010	0·023 ± 0·005
	Chlorpromazine	30		0·201 ± 0·006	0·023 ± 0·003
	Chlorpromazine	15		0·156 ± 0·009	0·021 ± 0·003
3	Control		2	0·191 ± 0·013	0·024 ± 0·003
	d-Amphetamine	2·5		0·176 ± 0·010	0·025 ± 0·004
4	Control		2	0·192 ± 0·005	0·029 ± 0·004
	d-Amphetamine	5		0·187 ± 0·006	0·030 ± 0·003
5	Control		2	0·198 ± 0·005	0·030 ± 0·004
	d-Amphetamine	10		0·163 ± 0·003*	0·028 ± 0·004

All values are the means (± S.E.M.) of 9 or 10 determinations. Significance of difference from control: *(0·01 > P > 0·001).

The suggestion, based on investigations on the liver,[5] of an association between NAD metabolism and the pharmacological activities of reserpine, has not been confirmed for brain.[2] Nevertheless, since massive doses of nicotinamide, sufficient to produce marked lethargy in experimental animals,[8] have been used to treat schizophrenia,[9] it is possible that the modification of NAD metabolism may be a factor in the action of psychotropic drugs. Supporting evidence has been provided by studies revealing a correlation between the behavioural effects of certain psychotropic drugs and their abilities to alter brain levels of adenine nucleotides,[10–13] which are involved in the metabolism of nicotinamide nucleotides.[14]

The results obtained with *d*-amphetamine (10 mg/kg) are compatible with those of Lewis and Van Petten,[10, 12] who showed that a number of behavioural stimulants, including *d*-amphetamine and iproniazid, increase brain ATP levels. However, lack of an effect in these preliminary experiments with chlorpromazine, which produces a fall in brain ATP,[15] prevents a comparable inverse relationship between brain ATP and NAD levels being postulated for this drug.

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EFFECTS OF DRUGS ON RAT BRAIN *IN VITRO* RESPIRATION AND ADENOSINETRIPHOSPHATASE ACTIVITY

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Increased brain ATP levels following the administration of amphetamine-like drugs and antidepressives may reflect an alteration in the dynamic equilibrium between utilization and resynthesis of ATP (1,2). Whether these drugs produce this response by an effect upon a specific regulating mechanism requires further investigation. The present study was carried out to determine if the raised ATP levels reported by Lewis and Van Petten (1,2) could be correlated with the modification of either the *in vitro* cortical respiration or ATPase activity of the rat brain. The drugs used were d-amphetamine, l-amphetamine, d-methylamphetamine, l-ephedrine, dl-phenmetrazine, iproniazid, isoniazid, dl-tranylcypromine and imipramine.

Methods

Male albino rats (150-200g) were stunned, exsanguinated and the brains excised and transferred to ice-cold phosphate medium, (3a) containing glucose (0.2%). The cerebral cortex from each brain was sliced to give 0.36 mm sections (4). Oxygen uptake was measured by the Warburg "Direct method" at 37°C in an atmosphere

of air (3b), readings being taken for 5 hours. For the determination of ATPase activity, rat brains were excised, chilled in ice-cold isotonic (0.25 M) sucrose and weighed. The tissue was homogenised (2 min., 1500 rev./min., 0°C) in 9 volumes of sucrose solution (0.25 M). In some experiments, cerebral cortex slices, prepared as above, were used. ATPase activity was determined in the presence of Ca^{++} and Mg^{++} (5) by the method of Lardy and Wellman (6). The inorganic phosphate liberated was estimated spectrophotometrically. (7).

Results

Only d-amphetamine (10 M) and imipramine (10^{-3} and 10^{-4}M) produced a significant inhibition of ATPase activity (Table 1).

Table 1

Effects on ATPase Activity

Drug	Concentration (Moles)	Inorganic Phosphate Liberated (Micromoles)
	Whole Brain Homogenate	
dl-Phenmetrazine	10^{-2}	2.203±0.060
	Control	2.209±0.071
dl-Tranylcypromine	10^{-3}	2.807±0.058
	Control	2.820±0.037
Imipramine	10^{-3}	2.088±0.088 *
	Control	4.134±0.113
Imipramine	10^{-4}	3.868±0.039 *
	Control	4.174±0.051
	Cerebral Cortex Slices	
l-Ephedrine	10^{-2}	1.482±0.074
	Control	1.472±0.068
d-Amphetamine	10^{-2}	1.350±0.010 *
	Control	1.572±0.010

The results are in micromoles of inorganic phosphate liberated (±S.E. of mean)/100 mg wet weight of tissue or 0.3 ml homogenate (10% W/V)/hour. The significance of the difference from the control mean is given by the P value ($P<0.001^{*}$, $0.01>P>0.001^{+}$, $0.05>P>0.01^{o}$).

The drugs inhibited *in vitro* respiration at higher concentrations than those likely to be found in the brain following a therapeutic dose. Most inhibited oxygen uptake at 10^{-3}M. However, the weak behavioural stimulants, isoniazid, ephedrine and phenmetrazine had no significant effects, even at very high concentrations. An exception was l-amphetamine, which was almost equipotent with d-amphetamine. Only imipramine increased oxygen uptake, an effect subsequently reversed in the remaining 3 hours (Table 2).

Table 2

Effects on *In Vitro* Respiration

Drug	Concentration (Moles)	Hour of Experiment 1	2	3	4	5
d-Amphetamine	Control	103.56 ± 5.66	93.73 ± 4.84	94.69 ± 2.82	101.82 ± 3.78	100.69 ± 5.21
	5×10^{-5}	105.38 ± 4.96	92.49 ± 4.52	92.84 ± 1.64	96.33 ± 2.56	98.06 ± 5.50
	5×10^{-4}	99.48 ± 4.11	80.15 ± 4.74	51.27 ± 2.38 *	35.13 ± 3.33 *	25.65 ± 2.53 *
l-Amphetamine	Control	92.20 ± 9.42	100.94 ± 1.44	114.89 ± 5.01	96.15 ± 5.04	98.22 ± 2.22
	5×10^{-4}	82.39 ± 16.62	94.35 ± 3.92	103.11 ± 1.93	83.93 ± 2.96	71.41 ± 3.96 +
	5×10^{-3}	83.72 ± 15.93	74.14 ± 2.19 *	51.84 ± 3.31 *	22.93 ± 4.69 *	11.44 ± 5.48 *
d-Methylamphetamine	Control	107.57 ± 2.71	93.62 ± 2.36	98.92 ± 2.73	94.51 ± 2.55	84.29 ± 2.74
	5×10^{-5}	105.95 ± 3.97	93.40 ± 1.37	96.78 ± 3.76	91.05 ± 2.45	80.57 ± 3.40
	5×10^{-4}	106.64 ± 2.15	94.15 ± 2.07	98.21 ± 2.12	84.78 ± 2.14 o	77.84 ± 2.50
l-Ephedrine	Control	93.19 ± 2.97	70.22 ± 2.19	88.95 ± 3.07	88.33 ± 4.24	76.22 ± 5.11
	10^{-5}	93.03 ± 3.68	76.65 ± 4.81	90.96 ± 4.85	90.80 ± 2.84	81.03 ± 2.44
	10^{-3}	94.48 ± 5.55	74.99 ± 3.91	85.91 ± 5.16	88.00 ± 2.46	70.54 ± 3.58
dl-Phenmetrazine	Control	104.82 ± 0.63	101.38 ± 1.37	108.21 ± 2.79	103.55 ± 3.00	116.07 ± 4.10
	10^{-5}	106.25 ± 3.25	102.32 ± 2.68	107.82 ± 2.64	103.83 ± 2.05	111.46 ± 4.67
	10^{-3}	109.20 ± 3.81	97.41 ± 2.57	98.54 ± 3.31 o	99.97 ± 2.63	109.42 ± 4.16
Iproniazid	Control	109.70 ± 4.75	91.03 ± 4.01	96.40 ± 6.17	85.89 ± 5.24	60.96 ± 8.19
	10^{-5}	108.44 ± 3.09	91.37 ± 2.69	93.32 ± 4.64	82.27 ± 4.90	58.78 ± 5.02
	10^{-3}	104.62 ± 2.99	92.86 ± 2.35	83.61 ± 5.58	67.73 ± 5.21 o	44.34 ± 7.02
Isoniazid	Control	87.62 ± 5.00	97.86 ± 2.43	108.53 ± 3.13	112.30 ± 3.53	106.81 ± 4.69
	10^{-5}	89.22 ± 6.19	93.68 ± 1.66	102.61 ± 1.83	107.81 ± 2.62	103.86 ± 5.45
	10^{-3}	90.80 ± 4.96	97.83 ± 1.74	104.21 ± 2.17	108.83 ± 2.25	107.01 ± 5.87
Imipramine	Control	90.44 ± 3.64	90.51 ± 1.29	99.46 ± 1.94	93.87 ± 4.13	104.56 ± 4.31
	4×10^{-4}	91.70 ± 4.02	110.12 ± 2.71 *	87.67 ± 3.50 o	53.33 ± 4.22 *	41.11 ± 6.10 *
	4×10^{-3}	93.83 ± 2.43	13.74 ± 1.36 *	3.57 ± 1.33 *	3.08 ± 0.42 *	5.88 ± 1.16 *

The results are in microlitres of oxygen absorbed (± S.E. of mean)/100 mg wet weight of tissue/hour. The significance of the difference from the control mean is given by the P value ($P<0.01$ *, $0.01>P>0.001$ + , $0.05>P>0.01$ o). The drugs were tipped into the main compartments at the end of the first hour.

Discussion

The intact brain derives its energy almost entirely from the aerobic oxidation of glucose. (8). Alteration in the functional activity of the brain may (8,9) or may not (10) be associated with altered cerebral respiration. Even drugs which modify central function without affecting overall brain oxygen uptake may exert a localised effect within the brain at a particular area or areas, so small as to have no effect on general brain respiration (8). The results obtained suggest however, that the drugs used do not act by modifying cerebral respiration.

The hydrolysis of ATP in the brain provides energy for the syntheses of glutamine, (11) noradrenaline, (12,13) and acetylcholine (14) and for the maintainance of ionic gradients.(15-18) Evidence suggests that the ATP-ATPase system is involved as a link between energy metabolism and the active transport of ions. (19-25) It has also been suggested (26) that modification of brain ATPase activity would provide an explanation of some of the pharmacological properties of reserpine and chlorpromazine and that the sustained fall in brain ATP caused by these drugs might be due to the modification of some homeostatic mechanism responsible for the control ATP levels. It is possible that ATPase may be involved in such a mechanism. However, the results of this investigation suggest that the inhibition of ATPase is unlikely to explain the increased brain ATP following treatment with d-amphetamine and dl-tranylcypromine. (1,2) Thus, whereas dl-tranylcypromine has no effect on ATPase activity and d-amphetamine produces a significant effect only at very high concentrations (10^{-2} M), imipramine, which is less active in increasing brain ATP, (2) inhibits ATPase at lower dose levels (10^{-3} and 10^{-4} M). These results agree with those of Lyons, (27)

who showed that imipramine inhibits liver mitochondrial ATPase. The *in vitro* ATPase inhibitory activity of imipramine may reflect an *in vivo* interference with the active transport of ions in the brain. On the other hand, since imipramine reduces the 5-hydroxytryptamine content of blood platelets, (28) it is also possible that it may affect the storage or transport of this or other amines in the brain.

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