

THE EFFECTS OF L-METHIONINE IN SCHIZOPHRENIA AND ITS
ROLE AS A METHYL DONOR IN THE METABOLISM OF BRAIN AMINES

by

F.T. ANTUN

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"It is better to light one candle than curse the darkness".

Motto of the Christopher Society -
Anna E. Roosevelt.

Statement in Terms of Ph.D. Regulation 13
of the University of Edinburgh.

1. The clinical and biochemical effects of L-methionine on chronic schizophrenics have been presented at a meeting in Dundee organized by the Scottish Psychiatric Research Society in 1970.

2. The role of L-methionine in donating methyl groups to catechol amine catabolites in vivo has been the subject of a paper presented at a symposium on "Brain Chemistry and Mental Disease" sponsored by the Faculty for Advanced Studies, Texas Research Institute of Mental Science. The meeting was held in Houston, Texas in 1970.

3. The following have been sent for publication:-
 - (a) Antun, F., Burnett, G., Cooper, A.J., Daly, R., Smythies, J.R., Zeally, A. The effect of L-methionine (without MAOI) in schizophrenia. In press - J. Psychiat. Res.

 - (b) Antun, F., Eccleston, D., Pullar, I.A., Sharman, D.F. A fluorometric method for the determination of 4-hydroxy, 3-methoxy phenyl glycol (HMPG) in urine. For publication in Clin. Chim. Acta.

SUMMARY

The thesis consists of three parts:

I. Review of the relevant Literature on -

- (a) Transmethylation and Schizophrenia
- (b) Biological Methylation

II. Human Studies: The effect of L-methionine feeding (without MAOI) on chronic schizophrenics.

Clinical

1. In a double blind cross over study of L-methionine against glycine (placebo) without MAO-inhibitors L-methionine produced a schizophreniform psychosis in nine out of sixteen schizophrenics. The psychosis was different clinically from a toxic psychosis which preceded it in two cases. Thus, chronic schizophrenics could be divided into two groups, "reactors" and "non-reactors" to L-methionine. However, no predictions could be made on clinical grounds as to whether a particular schizophrenic would react or not.
2. Following recovery from the immediate effects of L-methionine there was no significant change in the mental state and behaviour of any of the patients. L-methionine was neither harmful nor therapeutically beneficial.

Biochemical

1. A method for the determination of HMPG (4-hydroxy, 3-methoxy phenyl glycol), the major O-methylated catabolite of noradrenaline centrally, in urine was developed. HMPG and VMA (4-hydroxy,

3-methoxy mandelic acid), the major O-methylated catabolite of NA in the body, were estimated every other day in 24 hour urines of six male chronic schizophrenics over the twelve week period of the L-methionine study. The results showed that L-methionine did not produce a statistically significant increase in the urinary levels of either HMPG or VMA.

2. The 24 hour urinary levels of HMPG in the six chronic schizophrenics were compared with those from five normal volunteers and five acute schizophrenics. The levels in the acute schizophrenics were statistically higher than those in the chronic schizophrenics and normals; however, no statistically significant difference was found between the latter two groups.

III. Animal Studies

1. L-Methionine - a methyl donor for O-methylation of catecholamine catabolites, in vivo:

C¹⁴-methyl L-methionine was injected intravenously into two groups of rats. One group was killed two hours after the injection and their brains assayed, while in the other group 24 hour urine was collected for assay. Some of the C¹⁴ radioactivity detected both in brain and urine was identified, using different chromatographic procedures, as O-methylated catabolites of NA (noradrenaline) and dopamine. This may suggest that, in vivo, O-methylated catabolites derive their methyl groups from L-methionine.

2. The effect of L-methionine loading on noradrenaline catabolism in rat brain:

One group of rats was fed for four days and then injected intraperitoneally with L-methionine while another control group was treated similarly using glycine instead. Both groups were injected intraventricularly with C¹⁴-NA three hours after the intraperitoneal injection and killed one hour after the intraventricular injection. Brain assay for NA catabolites showed that L-methionine loading did not increase the levels of the O-methylated NA catabolites as compared to the non-O-methylated ones. The results showed similar percentages of the catabolites in both groups, except for DHMA (dihydroxy mandelic acid). The percentage of that catabolite was lower in the L-methionine group. However, it is doubtful whether this finding has any significance.

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Abbreviations and terms used frequently in the text

L - Levo isomer

D - Dextro isomer

DL - Both isomers

C^{14} - Carbon 14 radioisotope

H^3 - Tritium radioisotope

C^{14} -methyl - L-methionine - the active methyl group is C^{14} labelled.

MAO - Mono amine oxidase

MAOI - Mono amine oxidase inhibitor

COMT - Catechol O-methyl transferase

NA - Noradrenaline - norepinephrine

Epinephrine - Adrenaline

O-methyl - Methoxy group (CH_3O-)

N-methyl - Methyl group on nitrogen (CH_3N-)

NM - Normetanephrine

M - Metanephrine

MTYR - 3-methoxy tyramine - 3-methoxy dopamine

HVA - Homovanillic acid, 3-methoxy, 4-hydroxy phenylacetic acid

VMA - Vanylmandelic acid, 3-methoxy, 4-hydroxy mandelic acid

DHMA - Dihydroxy mandelic acid

DOPAC - Dihydroxy phenylacetic acid

HMPT - 4-hydroxy, 3-methoxy phenyl ethanol

HMPG - 4-hydroxy, 3-methoxy phenyl glycol

DHPG - Dihydroxy phenyl glycol

INTRODUCTION

For the past fifty years research workers have sought for some biochemical or biological dysfunction (or "lesion") in schizophrenia, without, until recently, any very encouraging results. Although it is generally agreed that the aetiology of the neuroses may lie in psychological and environmental factors, it has been widely held that schizophrenia and manic-depressive psychoses are associated with some error of metabolism; however, this is not to deny the subordinate importance of psychological factors which may well modify the development of the underlying biochemical abnormality.

In the past, biochemical research work on schizophrenia had been carried out on purely empirical lines. Generally there was a dearth of specific hypotheses and people merely applied the biochemical procedures that were available to them at the time.

Such research included studies of adrenocortical and liver functions, blood glucose and, latterly, serum aldolase and kinase, etc., without showing any conclusive and reproducible findings. During the last decade there has been a change in research strategies in the way of construction of several specific biochemical hypotheses; these include the mescaline hypothesis, the autoimmune theory, the abnormal phenylalanine metabolism hypothesis, the abnormal tryptophane metabolism hypothesis, the psychotomimetic indole hypothesis and the kynurenin hypothesis. However, most of the above hypotheses have failed to gain widespread support, due either to the incompetence or limitations of biochemical methodology, and/or to failure of replication by other workers.

An hypothesis that attracted some interest among research workers was the "Mescaline Theory"; this suggested that schizophrenia was a disorder involving the process of methylation in the body. In 1952 when Osmond, Harley-Mason and Smythies put forward this hypothesis, they focused attention only on disorders of methylation involving the catecholamines. This approach was limited because mescaline (an hallucinogen) has a related chemical structure to noradrenaline. In the course of time research work became more sophisticated and knowledge accrued especially in the area of amine metabolism in brain and periphery. Thus both O and N methylation of catechol and indole amines were confirmed as an important step in their catabolism, etc. These findings gave additional tentative support to the mescaline theory which by then was better known as the "Transmethylation Theory" of schizophrenia. The assumed importance of transmethylation was tested in a number of ways.

The present investigation is an extension of this work and in some respects is complementary; it consists of:-

1. A review of the relevant literature, both historical and more recent, which relates to the transmethylation theory and the role of L-methionine in catecholamine metabolism.
2. A detailed clinical study of the effects of L-methionine without MAO inhibitors on schizophrenic patients with two main objectives:
 - (a) To assess and characterize the so-called "Methionine Reaction" with special reference to its specificity.
 - (b) To evaluate the alleged therapeutic benefits of L-methionine in schizophrenia.

3. A biochemical study of the effects of L-methionine on catecholamine metabolism in schizophrenics, specifically a longitudinal study of the levels of normally occurring O-methylated catecholamine catabolites in the urine, with special emphasis on any changes in these during L-methionine administration.

4. Animal experiments designed to show in vivo:

- (i) that there is a transfer of methyl groups from L-methionine to the various O-methylated catecholamine catabolites.
- (ii) whether L-methionine loading increases the brain levels of catechol-O-methylated catabolites.

SECTION I

REVIEW OF THE LITERATURE

Transmethylation and Schizophrenia

Historical Background: Since the 19th century it has been held that model psychoses (schizophreniform psychoses produced by toxic substances) might give clues to the mechanism of the naturally occurring psychoses. Moreau de Tour (1845) published a monograph on the subject: "Du Hachich et de L'Alienation Mentale. Etudes psychologiques". Prentiss and Morgan, as long ago as 1895, pointed out that mescaline could induce a "toxic psychosis". Beringer (1927) reviewed the literature and published a monograph, "Der Meskalinrausch", in which he compared mescaline intoxication with schizophrenia, stating that a study of the effect of mescaline in non-schizophrenics might help in understanding disturbances in intermediary metabolism as a cause of schizophrenia. However, his report was limited to clinical interests and did not mention the similarity in chemical structure of mescaline (which was identified by Spaeth in 1918) and noradrenaline.

De Jong (1930 and 1945) in Holland was interested in the symptoms of catatonia. He completed a series of animal experiments and showed that several compounds, especially bulbocapnine, were capable of producing "experimental catatonia". He did not consider the states identical, but pointed out that the resemblance of experimental catatonia to schizophrenic catatonia might be important in understanding the aetiology of the latter. In 1931 he showed that experimental catatonia in different animal species could be produced by mescaline. He realised the similarity of chemical structure between mescaline and adrenaline and went further to show that adrenaline and also acetylcholine could both produce a "catatonic syndrome". Noteboom (1934) then investigated a series of mescaline and adrenaline derivatives including 3,4 dimethoxy-phenylethylamine (DMPEA) which produced "catatonia".

However, subsequently interest waned, especially among psychiatrists. This was probably due to the difficulty in the interpretation of the "catatonic state" in animals, the non-specificity and variability of the human catatonic syndrome and the unavailability of biochemical techniques to assess brain metabolism. Mayer-Gross (1951) discussed model psychoses and again drew attention to the similarity of chemical structure between mescaline and adrenaline.

The Mescaline Theory: Osmond and Smythies (1952) together with the chemist, Harley-Mason, advanced their hypothesis that schizophrenia

might be caused by a metabolic aberration in which mescaline-like metabolites of adrenaline could be formed in the body and thus produce somewhat similar psychological phenomenae as mescaline itself. At the time, little was known about the metabolism of adrenaline although a note by Harley-Mason was included that stated in part:

"It is extremely probable that the final stage in the biogenesis of adrenaline is a transmethylation of noradrenaline, the methyl group arising from methionine or choline. It is just possible that a pathological disordering of its transmethylation mechanism might lead to methylation of one or both of its phenolic hydroxyl groups instead of its amino group . . . Methylation of phenolic hydroxyl groups in the animal body is of rare occurrence but a significant case has been reported recently . . . It is particularly interesting to note that out of a series of phenylethylamine derivatives tested by Noteboom, 3,4 dimethoxyphenylethylamine was the most potent in producing catatonia in animals".

Osmond, Harley-Mason and Smythies suggested that the phenolic hydroxyl groups of adrenaline might be methylated so that a mescaline-like structure was formed and they considered that 3,4 dimethoxyphenylethylamine might be the substance involved. This hypothesis concerning "excess methylation" has since been useful in directing research on schizophrenia. In the light of the above remarks it is perhaps surprising that nobody studied the psychological effects of DMPEA in man before Hollister and Friedhoff (1966) who found it to be practically inactive.

O-Methylation of Catecholamines - the "Pink Spot": Armstrong et al (1957) showed that 3-methoxy-4-hydroxymandelic acid (VMA) was the main metabolite of noradrenaline and Axelrod et al (1959a) showed that O-methylation of noradrenaline and adrenaline was a major step in their degradation, although this was either preceded or followed by deamination. Later, other metabolites were detected (see review by Axelrod (1959)) and used as research "tools" in schizophrenia.

Bergsman (1959) showed that there were no differences between schizophrenic and normal persons in the excretion of adrenaline and noradrenaline in the urine. Also Mann and La Brosse (1959) and Pind and Faurbye (1961) found that both schizophrenics and normals excreted equal amounts of vanilylmandelic acid (VMA). Furthermore, La Brosse et al (1963), who infused tritium labelled adrenaline intravenously in twelve schizophrenic and twelve normal control persons, found no difference between the two groups in the level of adrenaline and noradrenaline in blood, taken at consecutive hourly intervals following the infusion. The two groups also excreted the same urinary amounts of the various catechol metabolites. There was, therefore, no evidence to indicate any abnormalities of catecholamine metabolism in schizophrenics until Friedhoff and Van Winkle (1962, 1962a) announced that they had identified 3,4 dimethoxy-phenylethylamine (DMPEA) in the urine of schizophrenics but not in the urine of normal controls. They also infused tritium labelled dopamine (the presumed precursor of DMPEA) in five schizophrenics, then collected their urine for the next three hours, and claimed that DMPEA, 3-methoxy, 4-hydroxyphenylethylamine, 3,4-dimethoxyphenylacetic acid and 3-methoxy-4-hydroxyphenylacetic acid were isolated (Friedhoff and

Van Winkel, 1963). Moreover, they reported that liver tissue from a schizophrenic patient could convert dopamine to DMPEA in vitro. This work, however, was not confirmed by Wagner et al (1966) or Faurbye and Pind (1967) who, in addition, were unable to either isolate DMPEA from the urine of schizophrenics, or find any difference in labelled dopamine metabolites between normals and schizophrenics after the infusion of labelled dopamine. They also showed that liver tissue from both schizophrenics and normals did not convert dopamine to DMPEA. Friedhoff and Furiya (1967) stated that they continued to find DMPEA only in urine from schizophrenics.

Bourdillon et al (1965) reported that 60 per cent of schizophrenics excreted the abnormal metabolite, the "Pink Spot", while only one out of 300 normals and non-schizophrenic psychiatric patients excreted it. Furthermore, they found that 80 per cent of patients diagnosed as "non-paranoid" or "Schneider-positive" excreted the compounds whilst only 10 per cent of those diagnosed as "paranoid" or "Schneider-negative" did so. These results were confirmed by Kuehl et al (1964) in the USA and by Sen and McGeer (1964) in Canada, while Takesada et al (1963) in Japan and Studnitz and Nyman (1965) in Sweden stated on the other hand that they found the compound in the urine from both normal and schizophrenic persons. The latter finding was confirmed by Creveling and Daly (1967), who positively identified small amounts of DMPEA in urine using mass spectrometry, and Kuehl et al (1966), who identified the acid metabolite in larger amounts; however, there was no difference between normals and schizophrenics. On the other hand, Perry et al (1964) in Canada and Faurbye and Pind (1964, 1966, 1967a) in Denmark reported

that they were unable to demonstrate DMPEA either in the urine of schizophrenic or in the urine of normal persons. Williams et al (1966), Boulton and Felton (1966), Bell and Somerville (1966), Nunn and Wheeler (1966), using other analytical methods, also questioned the occurrence of DMPEA in the urine of schizophrenics.

The discrepancy in these findings was perhaps due to (i) the different analytical procedures used by the various workers in isolating and identifying the "Pink Spot" and (ii) several substances, such as p-tyramine (Boulton et al, 1967); bacterial intestinal products and dietary factors (Perry et al, 1966); drug metabolites (Faurbye and Pind, 1966, 1967) which might have interfered with the identification of DMPEA. Finally, Watt et al (1969) pointed out that the appearance or non-appearance of the "Pink Spot" was dependent on such factors as urine volume and pH; these had been neglected by previous workers. Watt et al demonstrated that the substance responsible for the "Pink Spot" was n-acetyl cadaverine, presumably derived from gut bacteria and that schizophrenics did not differ from normals in their excretion of it. At present Friedhoff and Van Winkle are the only remaining workers who maintain that DMPEA is the substance responsible for the so-called "Pink Spot".

Even if DMPEA should be excreted by schizophrenics, presumably it plays no role in the aetiology of that disorder since Hollister and Friedhoff (1966), who gave either DMPEA (500-1000 mgm orally) or mescaline or placebo "blind" to thirteen volunteers, seven of whom previously suffered from a "schizophrenic reaction", found that only mescaline produced psychotic symptoms. Likewise, Shulgin et al (1966), who gave DMPEA to normal persons, could not detect any

psychotic symptoms in any of them. The only remaining role for this substance in schizophrenia is the remote possibility that it may be toxic to schizophrenics who may be unable to demethylate it properly (*vide infra*).

N and O Methylation of Catechol and Indole Amines: During the late fifties and early sixties, new methylating enzymes were being discovered by different research workers. Phenylethanolamine N-methyltransferase (Keller et al, 1950 and Axelrod, 1962a), which converts noradrenaline to adrenaline in liver, adrenal medulla and brain (Barchas et al, 1969; Ciaranello et al, 1969), and hydroxyindole O-methyl transferase (Lerner et al, 1959; Axelrod and Weissbach, 1960), which converts N-acetylserotonin to melatonin, being (in the present context) among the most significant. Both require S-adenosyl methionine as a methyl donor and are substrate specific. Perhaps the third and most interesting N-methylating enzyme which is less substrate specific was isolated from rabbit lung by Axelrod (1961, 1962b). In vitro, it could N-methylate tryptamine and serotonin to the psychotomimetic derivatives N, N-dimethyltryptamine and bufotenine respectively. The enzyme could also N-methylate B-phenylethylamine, tyramine, dopamine, normetanephrine, metanephrine and a variety of exogenous compounds such as mescaline, amphetamine, etc. Although this enzyme has not yet been found in man, it generated theories relating to the possible functional significance of these compounds in the pathogenesis of schizophrenia.

The Trans-Methylation Theory: The discovery that methylation was an important step in the catabolism of catechol and indole amines

broadened the vistas of the "mescaline theory" which then became better known as the "Trans-Methylation Theory" of schizophrenia. The new concept simply implied the possibility that schizophrenia was due either to an aberration of methylation in the body or a defect in demethylation. Indole metabolites, as well as catechol metabolites, were incriminated. The interest in the former was engendered by the discovery of many psychotomimetic methylated indole derivatives such as N, N-dimethyltryptamine, psilocin and psilocybin.

Bufotenine (N, N-dimethylserotonin) was first claimed by Fabing and Hawkins (1956) to have psychotomimetic properties. This, however, was later denied (Holmstead, 1967; Turner and Merles, 1959). Like the "Pink Spot", bufotenine was suspected by some authors (Bumpus, 1958; Fischer et al, 1961; Brune et al, 1963) to occur in urines of schizophrenics, but denied by others (Siegel, 1965; Runge et al, 1966; Sprince et al, 1963). Tanimurkai et al (1967, 1970) claimed to have developed a more sensitive method to detect small amounts of bufotenine and DMT (N, N-dimethyltryptamine) in urine and with their method analysed the urine of four chronic schizophrenic patients on a controlled diet which excluded all pre-formed catechol and indole amines. They discontinued all other medications and gave their subjects monoamine oxidase inhibitors (MAO-inhibitors) and L-cystein. The authors reported that excretion of bufotenine was less (1 mg/24 hrs) in the absence of MAO blockade than with it (2-5 mg/24 hrs). On the other hand DMT could be detected in small amounts only after MAO blockade. This was regardless of the presence or absence of L-cystein. They were also able

to correlate the levels of urinary bufotenine and DMT with the clinical condition of the patients, finding that during an active psychotic state both compounds were excreted in larger amounts in the urine. However, although the controlled diet excluded preformed catechols, it contained preformed N-methylated amines present in plant food, and unavoidably tryptamine which cannot be completely eliminated from the diet.

McIsaac (1961) pointed to the similarity in chemical structure of melatonin (a pineal hormone) and harmine (a hallucinogenic drug). He suggested that melatonin might be involved in schizophrenia. However, this idea lacked experimental support until Greiner and Nicholson (1965) reported that melanin was deposited to excess in certain post-mortem tissues derived from schizophrenic patients. In humans, melatonin inhibits melanin production and, therefore, decreases pigmentation. On these grounds the authors hypothesized that the normal route of synthesis of melatonin from serotonin was blocked owing to the congenital absence of a certain enzyme (an O-methyltransferase) and instead of melatonin, hallucinogenic compounds like harmine were produced. However, this hypothesis did not take account of the possibility (i) that the excess melanin might have been due to an abnormality of adrenaline metabolism, since melanin can also be formed from tyrosin, and (ii) that an abnormality of O-methyl transferase itself might have been the primary cause and the excess melanin (due to decreased melatonin) might have been secondary. Although the Greiner-Nicholson theory lacked cohesion, since harmine itself is an O-methylated compound, excess pigmentation

in schizophrenic patients had been reported by Shattock (1950). Marsden (1965), on the other hand, argued that the apparent increased melanosis in schizophrenic patients might reflect some disorder in dopamine and noradrenaline metabolism in the brain. His hypothesis stated that during the process of embryonic cerebral development, catecholamine synthesis might be diminished in the brain stem nuclei, and tyrosine metabolism might be diverted towards the formation of neuro-melanin rather than catecholamines.

Although DMT and bufotenine were claimed to be present in schizophrenic patients, and were reported to increase just prior to exacerbation of psychotic symptoms, this work needs further confirmation. These observations are important since Morgan and Mandell (1969) reported the presence, in rabbit brain, of an N-methyl transferase capable of converting tryptamine to the hallucinogenic compound DMT.

Attempts to influence Methylation Reactions in Schizophrenia:

Hoffer et al (1957) administered niacin and niacinamine (both methyl acceptors) to schizophrenics and reported varying degrees of improvement in their clinical states; these findings have not been independently confirmed to date.

Cantoni (1953) showed that L-methionine, the essential precursor of S-adenosylmethionine, transferred its methyl group to acceptor compounds in the process of transmethylation. Furthermore, Baldessarini and Kopin (1963) reported that feeding L-methionine (which is biologically more active than the D-form which is readily metabolised in the liver; Harper et al, 1970) to rats produced a significant increase in S-adenosylmethionine in liver and brain. In an

attempt to study the effects of raising brain amines in the brain, Pollin et al (1961) investigated the effects of oral administration of a series of amino acids and other compounds on chronic schizophrenic patients, pre-treated with the MAO-inhibitor iproniazid. The latter was given since animal experiments had shown that MAO-inhibitors increased (i) the brain content of amines by blocking deamination, the alternative catabolic step in amine catabolism, and (ii) increased the rate of methylation in the body (Baldessarini, 1967). Thus MAO inhibitors had the dual function of firstly protecting any methylated metabolites from degradation by deamination and secondly, increasing the rate of O-methylation of amines. They found that "marked psychotic" changes occurred with L-methionine (20 g/day) in four out of nine patients. The psychotic reaction disappeared abruptly upon withdrawal of L-methionine and in some cases unexpected clinical improvement was reported; this persisted for weeks or months. The authors could not, however, be certain whether the psychotic reaction was a "flare up of the schizophrenic symptoms" or merely a "toxic delirium". In contrast, loading with tryptophane produced a mild psychic reaction in seven of nine patients, mainly characterized by stimulation, euphoria and, at times, elation. Loading with glycine, phenylalanine, glutamine, histidine, tyrosine, 5-hydroxytryptophane, dopa, ammonium chloride and placebo did not induce any reaction during the continued treatment with the MAO inhibitor. These findings focused research interest on L-methionine and tryptophane.

Brune and Himwich (1962a) gave L-methionine and an MAO-inhibitor (isocarboxazid) to nine schizophrenic patients. They observed a

psychotic syndrome in seven patients which they considered as being a combination of (i) non-specific toxicity plus (ii) a recurrence and exacerbation of previous schizophrenic symptoms. Park et al (1965) gave seventeen schizophrenics L-methionine or glycine (an amino acid without methyl donating properties) and isocarboxazid; two became overtly more psychotic while a third reacted less strikingly to L-methionine. From this study and their review of the literature, the authors concluded that it was impossible to differentiate between non-specific toxic reactions and accentuation of the schizophrenic symptoms proper. The effect of L-methionine and MAO-inhibitors on schizophrenic patients was also confirmed by Kakimoto et al (1967) who reported the same clinical effects, but again were unable to elucidate the nature of the reaction. They measured normetanephrine (NM) and metanephrine (M) in the urine of their schizophrenics and found no increase in the levels of these O-methylated metabolites of noradrenaline and adrenaline on L-methionine loading.

On the other hand, Alexander et al (1963) gave L-methionine (20 g/day) or L-tryptophane (15 g/day) with or without tranlycypromine to six schizophrenics and two non-schizophrenics. The combination of L-methionine and tranlycypromine produced a psychotic reaction in five of the six schizophrenics but only behavioural changes in the non-schizophrenics (insomnia, alertness, fear and agitation). L-methionine alone, L-tryptophane and L-tryptophane with tranlycypromine did not induce a psychotic reaction in any of the patients who manifested only behavioural changes vide supra. It was concluded that a combination of L-methionine + MAO-inhibitors was necessary to produce a psychotic reaction in schizophrenics which was only suggestive of a

relapse of their illness. Furthermore, Berlet et al (1965) gave a mixture of L-tryptophane and L-methionine to eight schizophrenic patients for a period of thirty days; there was no worsening of their psychotic symptoms. This procedure, however, increased the urinary excretion of tryptamine, 5-HIAA and L-methylnicotinamide. Seven patients who tolerated 30-40 g/day of L-methionine under an MAO-inhibitor (tranylecypromine) cover had moderate to severe worsening of their schizophrenic symptoms.

Brune and Himwich (1963) substituted betaine (another methyl donor) for L-methionine; they reported exacerbations of schizophrenic symptoms.

All these workers referred to above conducted their experiments on the assumption that the reaction produced by giving L-methionine or betaine to schizophrenic patients was due to the methyl donating properties of these compounds. On the other hand, Haydu et al (1965) who reported the ability of L-methionine to exacerbate schizophrenic symptomatology, found an ameliorating effect from hydroxychloroquine. They argued that L-methionine, as a precursor of thiol groups, might be inducing this effect by its action on these groups, and that hydroxychloroquine produced its amelioration by having an opposite effect to L-methionine on thiol groups.

Another interesting finding which might support Haydu's hypothesis was reported by Greiner and Nicholson (1965). They put forward the melatonin theory of schizophrenia and suggested that inhibiting the copper-dependent enzyme tyrosinase by means of a low copper diet and penicillamine (a copper chelating agent) should alleviate schizophrenic symptoms. A clinical test of this hypothesis

led to the report that (i) schizophrenics improved clinically, (ii) there was a fall in the number of pigmented cells in the epidermis, and (iii) copper excretion increased. However, these findings were at variance with those of Hollister et al (1966) and Walshe (1967). Whether or not penicillamine has a beneficial effect on schizophrenic symptoms, it is interesting to note that its chemical structure is similar to that of L-methionine, and moreover it carries an active thiol group. Thus, its copper-chelating properties might have nothing to do with any possible clinical effects which may alternatively be connected with the complex of the thiol groups and transmethylation.

Other metabolic effects of L-methionine have been studied and the presently available data may be interpreted as follows:

(i) Giving excess L-methionine might have resulted in a disturbance of cell membrane transport and cell uptake of other amino acids (Kakimoto et al, 1967), e.g. dopa (Shah et al, 1968). Sprince (1967) suggested that, in vivo excess L-methionine might favour the channeling of tryptophane metabolism into indoleacetic acid production by inhibiting the nicotinic acid pathway, or stimulating those pathways leading to the formation of indoleacetic acid. It was further speculated that excess L-methionine and/or its metabolites might disturb tryptophane metabolism by (i) liberating free tryptophane from the bound state, and/or (ii) inhibiting oxidation reactions, and/or (iii) forming N-methylated metabolites, and/or (iv) forming O-methylated metabolites, and/or (v) forming alpha-methylated metabolites, and/or (vi) stimulating (and/or depleting) pyridoxal phosphate activity. The last possibility was later confirmed by Sprince et al (1969).

(ii) Methionine is the source of methyl groups for the methylation of DNA bases. Griffith and Mahler (1969) advanced a theory of memory based on Sussman and Sussman's theory of DNA "ticketing". According to the theory DNA bases were altered by methylation, and it was suggested that methionine deficiency might underlie memory disorders. Equally, excess methionine could have exerted its enhancing psychotic effects through a similar mechanism.

The methionine loading experiments referred to above were not without criticism since (i) procedures were not standardized; for instance, some used large doses of L-methionine (more than 20 g/day), whilst others gave it for only two to three days (Kakimoto et al, 1967). (ii) In all the experiments MAO-inhibitors were used which might have themselves induced a psychotic reaction in the schizophrenics, or indeed, in predisposed persons. Thus, Birkmayer (1966) reported that eight out of two hundred Parkinsonian patients given isocarboxazid (marplan) 30 mgm/day over a long period of time, experienced psychotic episodes. The phenomenology was similar in all eight subjects; they became anxious and suspicious, and had auditory hallucinations and ideas of reference. These symptoms subsided on stopping the MAO-inhibitor, and reappeared when treatment was resumed.

Attempts to Decrease Methylation: Because L-methionine and other methyl donors proved to have a deleterious effect on schizophrenic symptomatology, Berlet et al (1966) placed nine schizophrenic patients on a controlled diet with constant amounts of protein, and reduced amounts of both tryptophane and L-methionine; their intention was to improve their clinical state. Tranquillizers were stopped two

to four months prior to the investigation. After a first period of six weeks, the content of L-methionine and tryptophane in the food was reduced for twelve weeks and followed by a second control period of eight weeks. There was no improvement in the patients' clinical state during the twenty-eight weeks of the experiment. On the contrary, psychotic episodes preceded by loss of appetite and weight were observed in two of the patients during both the control periods, and especially during the experimental phase, when dietary L-methionine and tryptophane were restricted. In these two patients, creatinine and indoles, especially tryptamine excretion in the urine were paradoxically increased during the psychotic episode. Since there was a correlation between creatinine and tryptamine excretion irrespective of tryptophane and protein intake, the authors hypothesized that the deficient amino acid intake might have resulted in an increase of tissue protein breakdown, leading to increased creatinine, tryptamine and presumably other psychotomimetic indole derivatives; the latter produced the worsening in the clinical state.

An observation made by Bogoch (1957) is relevant to these findings. He assumed that schizophrenia was associated with abnormalities in the metabolism of aromatic compounds. On this assumption, he gave twenty-two schizophrenics a synthetic diet, essentially free of aromatic amino acids (similar to that used for phenylketonuria), for three weeks. Contrary to his expectation, none improved, and thirteen patients became clinically worse. When phenylalanine and tryptophane were added to the diet, these patients improved. He concluded that both phenylalanine and tryptamine metabolism were involved in the pathogenesis of schizophrenia.

In an attempt to deplete the brain of amines (serotonin, dopamine and noradrenaline) Brune and Himwich (1960) gave nine schizophrenic, four "oligophrenic" and two phenylketonuric patients reserpine (4 mgm/day) with a diet which was constant in protein content. After a first placebo period of twenty days, all patients received the reserpine for twenty days followed by a second placebo period of twenty days. Throughout the experiment, urinary tryptamine and 3-IAA (3-indole acetic acid) were determined. In those patients who had an uneventful clinical course, urinary tryptamine remained within the normal range. On the other hand, those patients (five schizophrenic and one "oligophrenic") who experienced exacerbations of their symptomatology during both placebo and reserpine periods showed an increase of two to three-fold in urinary tryptamine; there was a lesser but still significant increase in urinary 3-IAA. In these cases the increase preceded the mental disturbance, and lasted until it abated.

In order to investigate whether there was a correlation between the excretion of tryptamine and the severity of schizophrenic symptoms, Brune and Himwich (1962b) studied twenty patients who were placed on a constant protein diet (100 g protein/day) and given reserpine and placebo alternatively. It appeared that a strong positive correlation existed between the severity of schizophrenic symptoms (as rated by the authors) and the levels of urinary tryptamine. This correlation was not influenced by reserpine administration. They concluded that dietary indoles were not an important factor in causing these fluctuations in urinary tryptamine; however, they were undecided whether this correlation applied only to schizophrenic psychoses or to psychoses in general.

There was a similarity between the mental disturbances observed in the reserpine experiments and those witnessed during the experiments using an amino acid deficient diet. Although such disturbances occurred during the placebo or normal diet periods, they were more frequent during reserpine administration, or a deficient diet. Since some of the symptoms were not specific to schizophrenia, it seems unlikely that this was a true exacerbation of the schizophrenic process in all affected patients. Moreover, it is quite possible that schizophrenic exacerbations could have occurred as part of the natural history of the illness and not due to the reserpine or deficient diet. Although little is known about the effects of a deficient diet on the metabolism of brain amines, it is interesting to note that psychotic episodes were not witnessed in the Minnesota experiment (Keyes et al, 1950)* and German concentration camps (Helweg-Larsen et al, 1952), both of which shared the common variable of deficient dietary amino acids. As for tryptamine excretion: (i) no account was taken, by the authors, of the effect of urinary factors such as pH, on the excretion of tryptamine, and (ii) to my knowledge no study was done to see whether a correlation existed between creatinine and tryptamine excretion in normals.

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In spite of these methodological criticisms, the correlation between increased levels of urinary tryptamine and manifest psychoses has previously been reported by Berlet et al (1964) and needs further

* This experiment investigated the effects of semi-starvation on forty-three volunteers. The dietary content of amino acids and vitamins was reduced; during this period metabolic (e.g. hormonal, biochemical) and psychological changes were assessed in each patient and compared with a control period during which a normal diet was given.

confirmation.

Krakoff (1961) reported that methionine sulphoximine (MSO), a methionine antimetabolite, when given to normal people at a dose of over 200 mg/day, produced a "toxic" psychosis characterized by hallucinations, agitation and disorientation. The EEG changes were suggestive of an organic disturbance. These effects reverted to normal by giving methionine, or were prevented by giving L-methionine together with the MSO in a ratio of nineteen parts to one respectively. This isolated but interesting finding led Heath et al (1966) to investigate the effect of MSO on schizophrenic patients. He gave MSO to ten schizophrenics and eight controls. All the controls, except the ninth, who was given placebo, experienced various combinations of psychotic symptoms, including autism, thought block, disturbances of affect and some catatonic behaviour, paranoid symptoms, auditory hallucinations and association defects. The ten schizophrenic patients showed no worsening of their symptoms, and in fact some were apparently improved. The EEG was abnormal in the controls but normal in the schizophrenic patients. The authors concluded that, when methylation was reduced in normals, a psychotic state developed, while any reduction of methylation which might have occurred in the schizophrenics would have neutralised the presumed "overactive" methylating pool.

Abnormal Methionine Metabolism: It is well known that temporal lobe epileptics are especially prone to a schizophrenia-like psychosis (Slater et al, 1963). Reynolds et al (1966a, 1967) had suggested that in many such patients the psychotic illness might be precipitated

by anticonvulsant drugs partly mediated through disturbances in folic acid and vitamin B12 metabolism (Reynolds et al, 1966b). Folic acid acts as an acceptor and donor of single carbon units and is required for the synthesis of methyl groups and ultimately methionine. Vitamin B12 acts as a co-enzyme in the transfer of the methyl group from 5-methyl tetrahydrofolate to homocysteine with the eventual formation of methionine (Woods et al, 1965; Arnstein, 1965). These findings are linked with the general concept of "aberrant methylation" underlying schizophrenia although they are only suggestive, since folic acid and vitamin B12 deficiencies have not been seen to produce a schizophrenia-like psychosis generally. However, in a "predisposed individual" with a temporal lobe lesion, a psychosis may be induced which can, but not invariably takes a schizophrenic-like form. These observations do not support the findings in the MSO feeding experiments, where reduced methylation was associated with an amelioration of the schizophrenic process. They do, however, imply that abnormal methionine metabolism might be associated with schizophrenia. Further evidence in support of this premise came from:-

(i) The discovery in Belfast (Carson et al, 1963) of homocystinuria, an inborn error of methionine catabolism, in which the normal route (via cystathionine) is blocked by absence of the enzyme cystathionine synthetase. Methionine metabolism is diverted and homosyctine is formed, and excreted in the urine. Affected individuals, when fed methionine, showed an increase in plasma and urinary levels of methionine. This rise in urinary levels was five to eight times

higher than in normal controls. The disease is associated with mental subnormality but, more interestingly, in the original family studied the mother and several of her close relations apparently suffered from schizophrenia. Other reports came from Perry et al (1967), who found psychotic illnesses in two out of three homocystenuric families, and Spiro et al (1965), who reported that a chronic schizophrenic was later discovered to have homocystenuria. Welch et al (1969), working with the same patient, failed to detect DMPEA or 4MPE (4-methoxyphenylethylamine) in her urine or in that of her mother, who also suffered from schizophrenia. There has as yet been no systematic survey of the prevalence of psychiatric symptomatology in these families. These findings perhaps suggest that an intensive biochemical study of these patients, to determine whether or not they might show other disorders of transmethylation, is relevant. To date, to the best of my knowledge, no such study has been completed.

(ii) Israelstam et al (1970) demonstrated that schizophrenics, when given small doses of radioactive L-methionine (C¹⁴-methyl group), showed a slower clearing rate of the radioactivity, measured as expired C¹⁴O₂ over a period of two hours. Normal subjects, as well as remitted schizophrenics, had a faster clearance rate. Although the methods used failed to pinpoint the precise "methylation defect", Israelstam et al tentatively inferred from their data that in some way schizophrenics might either (i) have a larger methylating pool, or (ii) a decreased turnover of this pool. This work has yet to be confirmed by others.

From all these studies it appeared that a psychosis of some kind might result from either (i) "excess" methylation (L-methionine), or

(ii) "insufficient" (MSO). Detailed examination of the literature revealed that those authors who reported a psychotic reaction with methionine and MAO-inhibitors in schizophrenic patients were (i) unable to describe the reaction in definitive terms, and/or (ii) to assess whether or not it represented an exacerbation of the schizophrenic process, or was merely a non-specific toxic psychosis. Moreover, no verified reports were available on the effects of similar doses of L-methionine in normal people. However, L-methionine in lower dosages (≤ 10 g/day) did not produce a psychotic reaction in normal volunteers (Harper et al, 1970). In addition to the difficulties and discrepancies, so far research workers have been unable to demonstrate increased methylated metabolites, "normal or abnormal", in schizophrenics receiving L-methionine. In fact, the only evidence presently available which suggests that L-methionine is the major methyl donor in the body has been extrapolated from in vitro animal experiments. Similarly the experiments done in an attempt to reduce methylation by either decreasing L-methionine intake or blocking its action by MSO may be criticized; thus Berlet et al (1966) did not use normal controls and failed to test whether or not there was any evidence for decreased methylated metabolites in their patients. This latter point is especially important since the study was done on the stated assumption that decreased methylation might alleviate schizophrenic symptoms. Although MSO is an antimetabolite of L-methionine, its effects are not confined to blocking methyl transfer from L-methionine. Since L-methionine has other functions in the body, it cannot be assumed that MSO produces its effects by blocking methylation. Furthermore, the

Heath et al (1966) study did not mention anything about the state of consciousness and orientation of their subjects on MSO; however, they did state that memory was impaired and that the severity of the reaction was dose dependent. Like Berlet et al, they did not examine whether MSO administration resulted in a decrease in methylated metabolites. In the light of the procedural deficiencies discussed above, many more methodologically adequate experiments are necessary to supplement these early studies. For instance: (i) a closer examination needs to be made of the effects of L-methionine on the excretion of methylated derivatives, (ii) the alleged effects of betaine and MSO on schizophrenic patients need confirmation, (iii) the ameliorating effects of penicillamine, hydroxychloroquine and nicotinamide together with an examination of their alleged mode of action needs investigation, and (iv) intensive biochemical investigations are necessary on patients suffering from homocystinuria, with or without schizophrenia.

Defective Demethylation - a possible defect in schizophrenia:

Many years ago Slotta and Müller (1936) reported that the 2.3.4 analogue of mescaline, inactive in normal people, was more active than mescaline in schizophrenics. This work, which has not to date been repeated, is interesting because, if schizophrenia is genetically determined, it is more probable that an inborn error of metabolism is associated with the complete or partial lack of an enzyme than the presence of an abnormal (methylating) enzyme. In the case of schizophrenia the absence of a demethylating enzyme is a possibility consistent with the transmethylation theory. As mentioned earlier, Axelrod (1961, 1962b) and later Morgan and

Mandell (1969) reported the presence of a non-specific N-methylating enzyme in liver (Axelrod) and brain (Morgan and Mandell). If these or similar enzymes are found in humans, then it could be postulated that schizophrenics are unable to demethylate the resultant O and N methylated metabolites, some of which might be hallucinogenic. Demethylation is an important metabolic reaction in the body and there is evidence that humans can demethylate mescaline (Harley-Mason et al, 1958); the Slotta and Müller (1936) experiment suggests it would be informative to investigate the demethylating enzyme system in normals and schizophrenics. Direct confirmation of their experiment leads, however, to ethical difficulties since it is no longer regarded as justifiable to feed potentially hallucinogenic compounds to schizophrenic patients.

Smythies et al (1967a) studied the role of methoxy groups on the benzene ring and found that, with the exception of the 3,4,5; 2,3,4,5 and 2,3,4,5,6, all the possible methoxylated derivatives of phenylethylamine were non-hallucinogenic, probably because they are rapidly catabolized by amine oxidase. They showed that at least three methoxy groups with a 3,4,5 configuration were necessary for the hallucinogenic properties. The addition of extra methoxy groups on the 2 and/or 6 positions was seen to enhance these properties; this probably being due to the resistance of these molecules to attack by diamine oxidase, since it is known that any compound substituted in the 2 and 6 positions is not catabolized by diamine oxidase.

Similar work on methoxylated amphetamines was done in rats by Smythies et al (1967b). Shulgin et al (1969) also tested the

hallucinogenic properties and potency of some of these compounds in humans. In general it was found that certain configurations of methoxy groups rendered the molecule hallucinogenic. Shulgin found that 4-methoxy amphetamine and the 2,5; 2,4; 2,4,5; and 2,4,6 derivatives were significantly more hallucinogenic than mescaline. These findings suggested that more than one configuration might render the molecule hallucinogenic. The fact that the amphetamine molecule was more potent in this respect than the phenylethylamine analogue was due to the presence of an α -methoxy group on the former, which makes it resistant to amine oxidase.

In the light of these latter findings, Slotka and Müller's (1936) observations that the 2,3,4-methoxyphenylethylamine was active in schizophrenics but not in normals might be explained on the basis of a demethylating defect in the former group. It is feasible that normals could demethylate the 4 position rendering the molecule inactive (since the 2,3 compound is inactive, while the 3,4 is very weakly active and the 2,4 is very active), whilst schizophrenic patients could not. Both methylation and demethylation are normal biochemical processes in the body; disturbance(s) of either could conceivably be involved in the casual mechanism(s) of schizophrenia.

This hypothesis can be tested by feeding small amounts of the 2,3,4 compounds C^{14} labelled on the various methyl groups and measuring the rate of demethylation in normals and schizophrenics.

Biological Methylation

For a long time "biological methylation" was thought to be a widespread biochemical process in the body with an important role in the metabolism of various compounds. Since 1950 important developments have taken place:

- (i) The discovery and isolation of S-adenosylmethionine as a prime methyl donor in transmethylation (Cantoni, 1951a, 1951b, 1952, 1953).
- (ii) Assessment of de novo methyl group formation from "active" formaldehyde in the biosynthesis of the methyl groups of thymine and methionine (Greenberg, 1963).
- (iii) The discovery that folic acid and Vitamin B12 were important in methyl group transfer (Guest et al, 1962a; Foster et al, 1964).

De Novo Methyl Group Synthesis: Thymine and methionine methyl groups are the only two groups which are definitely known to be derived by de novo synthesis from "active" formaldehyde (methylene-tetrahydrofolic acid). The mechanism in the two reaction sequences are distinctly different (Greenberg, 1963). The first in vitro experiment on methionine synthesis was by Berg (1951, 1953) who showed that methionine could be formed from homocysteine and formate. Later, Nakao and Greenberg (1955, 1958) identified most of the components and cofactors required for its synthesis. They found that using a liver enzyme preparation with homocysteine as a methyl acceptor, formaldehyde or the B-carbon of serine was converted to

the methionine-methyl group. The reaction required folate - H_4^* , ATP**, Mg^{++} and NAD*** or NADP****. This finding was confirmed by Doctor et al (1957) who also observed that the reaction rate was reduced when folic acid was omitted or aminopterin (a folic acid antimetabolite) was added to the preparation. Adenosyl homocysteine was the only substitute for homocysteine in stimulating the reaction (Stevens and Sakami, 1959; Wilmanns et al, 1960); it was formed from homocysteine and adenosine through methionine synthesis (de la Haba and Cantoni, 1959). However, more recent work suggested that under certain conditions Vitamin B12 was required for the synthesis (Foster et al, 1961; Kisluik and Wood, 1960; Guest et al, 1962b) and N^5 methyl-folate- H_4 was the actual methyl donor (Hatch et al, 1961; Larabee et al, 1961). It therefore appeared that there were two pathways for methionine synthesis, one of which was Vitamin B12 dependent. The present state of knowledge about methionine-methyl biosynthesis is that: homocysteine is the methyl acceptor and N^5 methyl-folate- H_4 serves as the methyl donor in an enzymatic reaction which is mediated through a methyl transferase; the reaction requires Vitamin B12, $NADH^x$, FAD^{xx} , ATP and Mg^{++} as additional cofactors (Diagram 1). Other methyl transferases can convert homocysteine to methionine with dimethyl thetin, methyl methionine, or betaine as methyl donors (Klee et al, 1961; Ericson, 1960; Durell et al, 1962).

* Folate - H_4 -5,6,7,8 - tetra hydropteroyl glutamic acid.

** ATP - Adenosine triphosphate.

*** NAD - Niacinadenine dinucleotide.

**** NADP - Niacinadenine dinucleotide phosphate.

^x NADH - Niacinadenine dinucleotide (reduced form).

^{xx} FAD - Flavin adenine dinucleotide.

Transmethylation: Transmethylation in the body is a widespread biochemical process which is involved in the metabolism and/or catabolism of many compounds. It takes the form of (i) N-transmethylation, (ii) O-transmethylation, (iii) S-transmethylation, and (iv) C-transmethylation. There is evidence for all forms of transmethylation with perhaps some doubt concerning the identity of some of the methylated products. It was found by Cantoni (1951a,b; 1952; 1953) that S-adenosylmethionine was the co-enzyme for most methyl transferase reactions. He established that ATP and Mg^{++} were essential for all transmethylation reactions involving methionine (Cantoni, 1951a,b) and were related to the formation of "active" methionine, subsequently identified as S-adenosylmethionine. The transmethylation activity of the latter resulted from an energy rich bond between the methyl group and sulfonium of the compound leading to labilization of the methyl group.

Of the many transmethylation reactions involving S-adenosylmethionine are the N- and O-methylation of catechol and indole amine derivatives. The former type of methylation was proved by Keller et al (1950) who, by feeding C^{14} -labelled-methyl group-methionine, demonstrated that norepinephrine was converted to epinephrine with the incorporation of the methyl group from methionine. The enzymic aspects of the reaction have been studied by Kirshner and Goodall (1957) who found that S-adenosylmethionine was the essential methyl donor in the reaction. Since then a number of N-methyl transferases have been discovered which include the indole - N-methyl transferases.

Indole and Catechol O-Methyl Transferase Enzymes: The only indole O-methyl transferase so far identified is acetylserotonine O-methyl transferase which is found in the pineal gland and converts acetylserotonine to the hormone melatonin in the presence of S-adenosylmethionine (Lerner et al, 1960; Axelrod and Weissbach, 1960).

The most exhaustive work done on catechol O-methyl transferase (COMT) was by Axelrod and Tomchick (1958) and Pellerin and D'Iorio (1958). The enzyme was found to methylate the 3-hydroxy position of catechol derivatives and, when further tested, could methylate all 3,4 dihydroxyphenyl derivatives including 3,4 dihydroxybenzoic acid and catechol itself, but not monohydroxy phenols. It was found in liver, brain and, to a lesser extent, in kidney of most mammals and was specific for S-adenosylmethionine with metal ion requirement for mg^{++} . Axelrod (1957) and Axelrod et al (1958) established that O-methylation of catechol amines occurs either prior to or after oxidative deamination and is the principal route for their catabolism. In later experiments Kopin et al (1961), using dual labelled isotopes (C^{14} -NA, H^3 -NM), showed that NA and NM were further catabolized in the body by deamination and O-methylation (Diagram II). The former route was blocked by MAOI and the latter by pyrogallol (a COMT inhibitor). Such evidence was obtained from the difference in the levels of O-methylated metabolites found in the three groups of rats (first group pretreated with MAOI, second group pretreated with pyrogallol, and third group served as a control). This work was later confirmed by Axelrod et al (1965).

DIAGRAM I

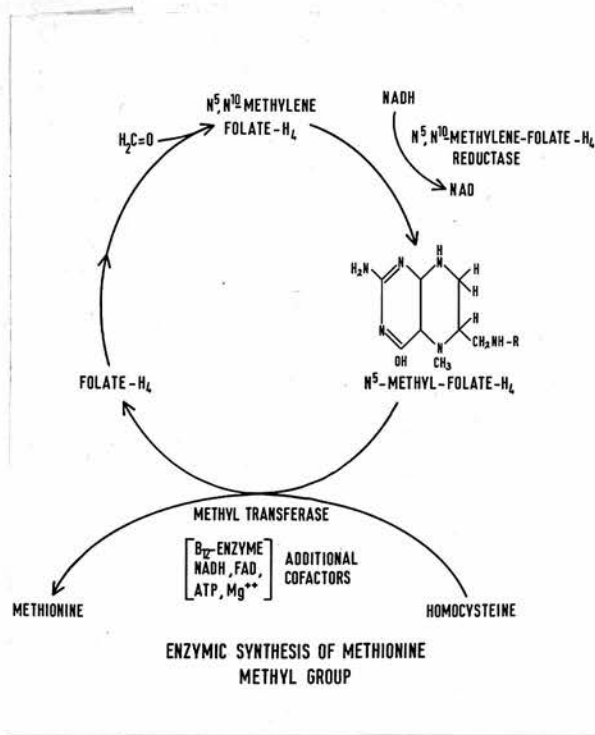
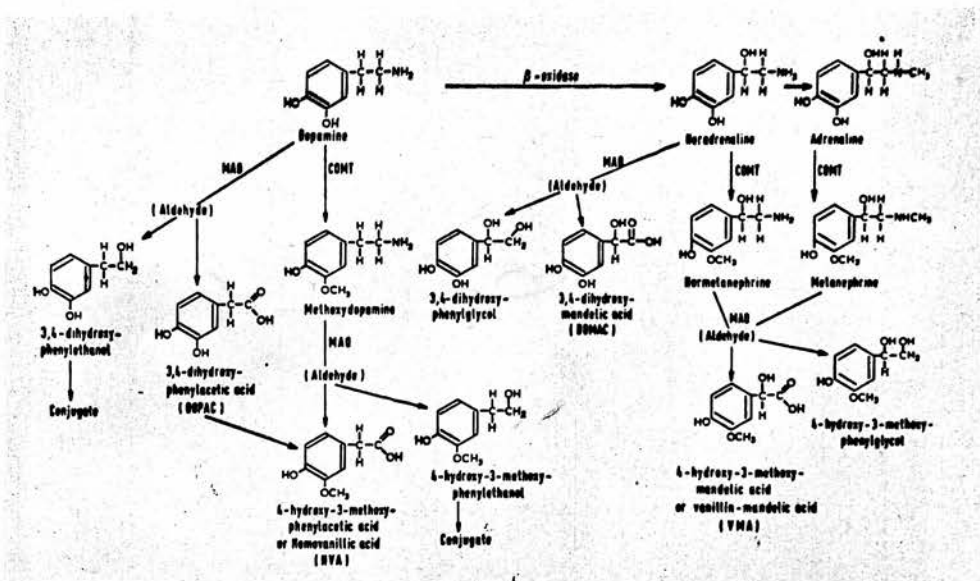


DIAGRAM II



METABOLIC PATHWAYS OF DOPAMINE AND NORADRENALINE

The role of COMT in the catabolism of catecholamines was also studied by the use of blocking agents such as pyrogallol and tropolones. Both act as competitive substrates for COMT (Archer et al, 1960) and the latter also specifically inhibits COMT, probably by complex formation with COMT active sites (Belleau and Burba, 1961). It was found that COMT inhibitors decreased the levels of O-methylated catechol metabolites in vivo (Carlsson and Waldeck, 1963; Carlsson, 1964; Kopin et al, 1961) and potentiated the effects of NA on adrenergic receptors (Bell, 1967; Belleau and Burba, 1963). More recent work (R. Sugden - personal communication, 1970) showed that when rats were injected intraventricularly with pyrogallol and C¹⁴-NA, the ratio, in brain, of 3,4-dihydroxyphenylglycol (DHPG) to 4-hydroxy, 3-methoxy phenylglycol (HMPG) increased as compared with the control group of rats where no pyrogallol was used. Furthermore, the increase in ratio was due to a significant decrease in HMPG accompanied by only a slight increase in DHPG.

Although all these findings established that catechol amines were partly catabolized by O-methylation and that S-adenosylmethionine was the co-enzyme for COMT, which was the specific enzyme for the reaction, no in vivo experiment was done to demonstrate the transfer of the methyl group from S-adenosylmethionine to the O-methylated catechol catabolites in brain. In all the experiments on O-methylation, the enzyme preparation was made from liver tissue and O-methylation was demonstrated, in vitro, by incubating the enzyme preparation with the appropriate substrates. The resultant labelled O-methylated metabolite (i.e. NM) was then

injected into the animal and its O-methylated catabolites were isolated (i.e. VMA, HMPG). Up to date, there is, therefore, no direct proof that, in brain, there is a transfer of methyl groups from S-adenosylmethionine to catechol-O-methylated catabolites.

VMA and HMPG - Two Major Catabolites of NA: Armstrong et al (1957) showed that 3-methoxy-4-hydroxy mandelic acid (VMA) was the main O-methylated catabolite of noradrenaline (NA) in the body. However, it was later discovered that the major O-methylated catabolite in rat (Schanberg et al, 1968, 1968a) and cat (Mannarino et al, 1963) brains was 4-hydroxy-3-methoxy phenyl glycol (HMPG). Moreover, Schanberg et al (1968) reported that in rat brain the majority of HMPG formed from exogenous labelled NM was identified as HMPG sulphate conjugate. However, this is not true for all species: whereas Schanberg et al (1968a) found that in rat brain and human CSF the majority of endogenous HMPG is conjugated, in the cat HMPG is mostly free and in the Rhesus monkey all HMPG is free. The detection of endogenous free and conjugated HMPG in brain was also confirmed by Sharman (1969). Mass and Landis (1966 and 1968), using double isotope technique in the dog, found that the major catabolite of brain NA excreted in urine was HMPG. It was concluded that in the dog 50-60 per cent of the endogenous NA catabolized in the brain was excreted in urine as HMPG.

It would appear from these findings that, in mammalian species, HMPG was the major NA catabolite in brain. As a consequence of this, depending on the size of the brain pool of NA and the amount of HMPG produced by peripheral stores (La Brosse et al, 1961;

Axelrod et al, 1959b), the catabolism of NA by the brain might account for a significant amount of the HMPG in the urine. Approximately 25 per cent of HMPG excreted in urine came from brain pools of NA (Mass and Landis, 1966).

In contrast to this, most of the normetanephrine (NM) and VMA excreted in the urine comes from peripheral NA stores rather than brain NA stores for the following reasons:- In different species there is a barrier to the movement of NM out of the brain (Mass and Landis, 1966; Schaneberg, 1967; Glowinski et al, 1965). Although this barrier may be complete or partial, it is assumed that little of the NM formed in brain enters the body pool and that the NM found in urine reflects the metabolism of NA by tissues other than brain. VMA, on the other hand, can be formed in brain with no existing barrier to its movement from it (Mannarino, 1963; Mass and Landis, 1966, 1968; Glowinski and Iversen, 1966). However, being the major NA catabolite in the body of most species, the brain's contribution to the amounts excreted in urine will be very small. It was found in the dog that less than 1 per cent of urinary VMA had its origin in brain (Mass and Landis, 1968).

Although accurate estimates of the different catabolites of NA in brain are not available, if man is similar to other species, it is probable that a significant amount of the HMPG excreted in urine comes from brain, whereas urinary VMA, metanephrine and possibly NM come from peripheral NA stores.

Factors Affecting S-Adenosylmethionine Levels in the Body:

Baldessarini (1967) has shown that a number of compounds affected tissue levels, including brain, of S-adenosylmethionine. In general, it was found that compounds which enhance methylation, i.e. MAOI and methyl acceptors, lowered the tissue levels of S-adenosylmethionine; on the other hand, methionine increased its levels considerably and this effect was reduced when MAOI were used together with the methionine. The distribution of S-adenosylmethionine in brain was found to be fairly uniform. Mackenzie et al (1950) studied the rate of oxidation of the methyl group of dietary methionine but were uncertain whether oxidation of the methyl group was associated with the activation of methionine to S-adenosylmethionine or its transfer to methyl acceptors. By giving C¹⁴ methyl-methionine to rats, they found that the oxidation of the methyl group (as measured by the C¹⁴ activity, in the expired breath, urine and faeces) took place in phases: the first phase, "assimilation phase", where there was a rapid decrease in radioactivity in expired breath about two hours after oral administration of methionine and one hour after its intraperitoneal injection. There was first a mobilization of already existing S-adenosylmethionine in tissue prior to the incorporation of the administered methionine. The second phase, "equilibrium phase", consisted of a steady and gradual fall in radioactivity, mainly in urine and faeces, which represented a steady incorporation and/or utilization of the methyl group from tissue methionine and/or S-adenosylmethionine. Moreover, Baldessarini (1967) showed that, with intravenous injection

of C¹⁴-methyl methionine, there was a rapid formation of S-adenosylmethionine from methionine in brain and liver; this was accompanied by a rapid turnover of S-adenosylmethionine in liver.

In the light of these findings, it seems possible to study, in animals, the effect of L-methionine loading on the rate of methylation of brain catechol amines. This would provide a direct evidence whether L-methionine, when given in excess, causes an increase in the levels of brain catechol-O-methylated catabolites.

SECTION II

THE PRESENT STUDY

B A C K G R O U N D

During the past decade the transmethylation theory has aroused general interest and some concomitant research has been carried out. Workers have taken various methodological approaches with the common aim of investigating transmethylation processes in schizophrenia. Although findings have varied and have been generally inconsistent, one definite and replicated observation has been the occurrence in schizophrenia of a psychotic reaction with L-methionine. Claims have also been made that this reaction may be followed in some by an improvement in their mental state.

The dearth of studies in the literature suggested the relevance of a systematic investigation into (i) the effects of excess L-methionine on schizophrenics and their methylation reaction, and (ii) the role of L-methionine, as a methyl donor, in the metabolism of catecholamines in vivo.

A I M S

There were four main objectives in the study:

- (a) A critical clinical evaluation of the effects of L-methionine (without MAO-inhibitors) on schizophrenics. (This evaluation was designed to observe closely the clinical symptomatology during L-methionine feeding and thereby attempting to differentiate a "toxic psychosis" from a schizophreniform psychosis.)

- (b) To investigate the claims made by previous workers that schizophrenics who manifested an L-methionine reaction showed long term improvement of their symptoms.
- (c) To conduct a longitudinal biochemical investigation into the effect of L-methionine on the body methylating pool in schizophrenics. This was done by measuring urinary levels of the two major O-methylated catabolites of noradrenaline, HMPG and VMA.
- (d) To demonstrate, using in vivo animal experiments:
 - (i) that L-methionine donates methyl groups to the O-methylated catabolites of dopamine and noradrenaline.
 - (ii) whether L-methionine loading increases the brain levels of catechol-O-methylated catabolites.

SECTION III

HUMAN STUDIES

THE EFFECT OF L-METHIONINE FEEDING (WITHOUT MAOI) ON CHRONIC SCHIZOPHRENICS.

M A T E R I A L S A N D M E T H O D S

(A) CLINICAL

(1) SELECTION OF PATIENTS

(i) PILOT STUDY: Five schizophrenic female patients were selected from the hospital wards. They were all under the age of 65 years and were not suffering from any other physical illness or psychiatric illness. The diagnosis of schizophrenia was confirmed by two psychiatrists.

(ii) MAIN EXPERIMENT: Eleven chronic schizophrenic patients from the hospital wards, without physical illness or other psychiatric disorder, were included. Their ages ranged from 46-60 years. Five of them were females with ages 46-60 years and six were males (ages 49-58 years). Leucotomized patients were excluded. For the purpose of (future) comparison their hospital course up to the time of the study was carefully charted for specific phenomena, relapses, remissions and any possible precipitating causes. Emphasis was placed on the degree of fluctuation in their mental states during the year before selection. Response to psychotropic drugs was taken into account and the patients taking minimal dosages of phenothiazines or butyraphenones were selected; seven patients were not receiving any drugs at the time of study. Patients who were not mentally deteriorated and exhibited subacute symptoms were preferred. The diagnosis of schizophrenia was confirmed by two psychiatrists. After

Why.

their selection and prior to the experiment, full explanations were offered to patients and relatives; subjects were only included if informed consent was obtained.

(2) DESIGN OF THE EXPERIMENT

The patients in the pilot experiment were studied in their ward, while those in the main experiment were transferred in two groups (5, 6 - the first group were females and the second males) to a special six bedded research ward. Two qualified nursing staff were with them at all times to ensure close supervision and assessment of ward behaviour. The patients were encouraged to participate in occupational therapy, some of which was carried out in the ward. Entertainment was organized so that the patients would not feel bored and isolated. They were allowed visitors within the time limits allocated by the hospital authorities. An environment resembling that of their own wards was sought in order to minimize social and environmental factors that might modify or alter their behaviour.

The patients (except the second group of six patients) received a normal diet which was standardized, and were allowed fluids ad lib. The second group received a special diet (vide infra).

The patients in the main experiment were transferred to the research ward for a period of twelve weeks. The design of the experiment, for both pilot and main experiment groups, was a double blind cross over of L-methionine (without MAOI) against glycine as follows:-

1. 4 weeks' observation, of which the first 2 weeks were used for acclimatization to the "new" ward and the second 2 weeks for rating.
2. 2 weeks of amino acid A.
3. 2 weeks of observation.
4. 2 weeks of amino acid B.
5. 2 weeks of observation.

The rating psychiatrists were not aware of the sequence in which the amino acids were given and, to protect against chance identification, were not allowed to handle them or witness their administration. Another psychiatrist, not involved with the experiment in any capacity, held the key to the experiment. The nursing staff, also blind, were only advised that a "reaction" might follow the amino acid administration. All medications were stopped six weeks prior to the administration of amino acid "A". This drug free period provided the base line for clinical and biochemical evaluation.

(3) ADMINISTRATION OF THE AMINO ACIDS

Both amino acids were given in the crystalline form dissolved with milk and Ribena (a blackcurrant cordial). The milk minimized any possible gastric irritation that might be caused by L-methionine (Reading, W., unpublished observation) and the Ribena masked the odor and taste of the amino acids. The L-isomers of both amino acids were given and the crystalline form was preferred to the tablets or capsules because of better absorption of the former. Both amino

acids were administered at a daily dose of 20 gms. for the first week and a daily dose of 10 gms. the second week. Each daily dose was given in three divided portions.

(4) PSYCHIATRIC ASSESSMENT

(i) Physician's Rating: The patients were rated every other day by two psychiatrists independently, using the "Lorr" rating scale (Lorr et al, 1962), a standard validated instrument for assessing schizophrenia. However, additional information relating to toxic effects not adequately covered on the scale were sought by an appropriate daily clinical interview. During these interviews, special attention was given to thought content, affect, orientation and state of consciousness; physical signs like vestibular and cerebellar functions were also assessed. The interviews and ratings were carried out twice a week for one year follow-up period to assess the patients' long term mental state.

(ii) Nurses' Rating: The patients were rated daily by the nursing staff, using the Henry Phipps ward behaviour rating scale. In addition, a detailed description of the patients' activities and behaviour was charted daily. This included the patients' interests and participation in the various ward activities, and was amplified by reports about their progress in occupational therapy. Daily meetings among the relevant clinical personnel ensured maximum useful data about each patient.

(B) B I O C H E M I C A L

(1) HMPG (4-hydroxy, 3-methoxy phenyl glycol)

Since the discovery of HMPG as a major O-methylated metabolite of NA in brain, several investigators attempted to measure it in brain or urine. Because most of the HMPG found in human cerebrospinal fluid (CSF) is conjugated in the sulphate form (Schanberg et al, 1968a), hydrolysis of the conjugate is essential prior to its determination. Two of the methods developed for its estimation (Ruthven and Sandler, 1965; Nicholas et al, 1969) used column chromatography (a cation exchange resin) to separate the HMPG conjugate, which was then hydrolysed, converted to vanillin and determined by spectrophotometry. Recoveries were low and the sensitivity of the method was limited by the interference of p-hydroxy phenolic acids. Another method used column chromatography (an anion exchange resin) to separate the hydrolysed HMPG, which was estimated by gas liquid chromatography (Wilk et al, 1967). Gas chromatography was also used by Sharman (1969) to identify hydrolysed HMPG, which was separated by paper chromatography. Although the Wilk et al method had reasonable recoveries (50-60 per cent), and sensitivity and specificity were good, it was time consuming, especially if the method had to be used routinely.

It seemed mandatory to develop a relatively simple and short, yet sensitive and specific, method for measuring HMPG in urine. This was of interest since it had to be used for a longitudinal study of the excretion of HMPG in the urine of patients; thus performed every other day. Sensitivity and specificity were

important because absolute levels of HMPG, as well as changes in them, had to be assessed. The present method was developed to measure urinary conjugated HMPG and it involved enzyme hydrolysis of the HMPG conjugate followed by separation of the hydrolysed HMPG by column chromatography (an anion exchange resin) (Wilk et al, 1967). The isolated HMPG was then measured by spectrophotofluorometry using the ferric chloride oxidation reaction developed by Sharman (1963) for the estimation of homovanillic acid (HVA).

(i) MATERIALS

HMPG was obtained from the Sigma London Chemical Company. All chemicals were "Analar" BDH grade, or purer. The resin was Bio-Rad AG1-X4, 200-400 mesh in the chloride form (Calbiochem). "Helicase" enzyme (Industrie Biologique Francaise) which contained 1,000,000 units (Fishman) of B-glucuronidase and 15,000,000 units (Roy) of sulphatase per gm. Glass distilled water was used throughout.

(ii) LABORATORY PROCEDURE

(a) ISOLATION OF HMPG

A 5 ml. aliquote of 24 hour urine was adjusted to pH 1 with conc. HCl and the organic acids present were extracted by shaking the sample with 10 ml. of ethyl acetate for five minutes. After centrifugation at 2,000 g for five minutes, the organic phase was removed and the extraction repeated twice with two further 10 ml. quantities of ethyl acetate. The pooled ethyl acetate was saved

for the determination of VMA. To 3 ml. of the aqueous phase, 0.3 ml. of 1M sodium acetate buffer pH5, was added, and the pH adjusted to 5 with 1N NaOH (glass electrode). The sample was then incubated in a water bath at 37°C for 24 hours with 27 mg. "Helicase" enzyme (9 mg/ml. urine). In order to prevent any bacterial decomposition occurring during hydrolysis, 2 drops of chloroform were added to each tube prior to incubation.

The hydrolysed sample was centrifuged for five minutes at 3,500 g and the supernatant removed. The residue was washed with 0.5 ml. distilled water and centrifuged. The combined supernatants were adjusted to pH6 with 1N NaOH and passed over a column (11 x 0.7 cm.) of Bio-Rad AG1-X4 resin. The resin was washed with 6 ml. water and the HMPG eluted with 12 ml. water. The eluate was saturated with sodium chloride and extracted twice with 12 ml., and a third time with 6 ml. of ethyl acetate. The combined ethyl acetate extracts were blown down to damp dryness with nitrogen at room temperature. The sides of the tube were washed with 3 ml. water, 2 ml. of which were taken for the determination of HMPG by fluorometry.

(b) FLUOROMETRIC METHOD

The reaction to produce the fluorophor from HMPG was carried out in 15 ml. glass stoppered centrifuge tubes. Because of light sensitivity (i) the reaction was carried out in a dimly lit room, and (ii) the test tubes were placed in racks covered with black paper. The FeCl₃ reagent was freshly prepared, prior to each batch of estimations, by adding 0.1 ml. 2N NaOH to 5 ml. 1% FeCl₃ solution

freshly diluted from 60% solution. The mixture was shaken thoroughly. The precipitate of ferric hydroxide formed a dark brown colloidal solution. To the 2 ml. sample containing HMPG was added 0.2 ml. of the ferric chloride reagent. The mixture was shaken thoroughly and the reaction allowed to proceed for exactly two minutes. At the end of that period, the reaction was stopped by the addition of 0.2 ml. 5N NaOH and the precipitate of ferric hydroxide removed by centrifugation at 5,000 g for one minute. The supernatant was transferred to a 3 ml. cuvette and the fluorescence estimated by activation scans on a Ferrand spectrofluorometer. Fluorescence maxima occurred at 325 m μ activation and 430 m μ emission (uncorrected values) (Figure 1).

A blank was prepared by taking 3 ml. distilled water through the extraction and fluorometric procedures. Recoveries were assessed by processing duplicate samples of urine, to one of which was added 5 μ g HMPG after the first ethyl acetate extraction, but prior to the treatment of the sample with "Helicase". Another blank of 2 ml. water was used for the external standard (5 μ g HMPG in 2 ml. water).

(iii) RESULTS

(a) THE SPECIFICITY OF ISOLATION PROCEDURE

The substances found in urine giving a positive reaction with the ferric chloride oxidation include the O-methylated catechols. Of these, $90 \pm 5\%$ (4) (mean \pm SD (no. of observations)) of the acids, 3-methoxy-4-hydroxymandelic (VMA) and 3-methoxy-4-hydroxy-

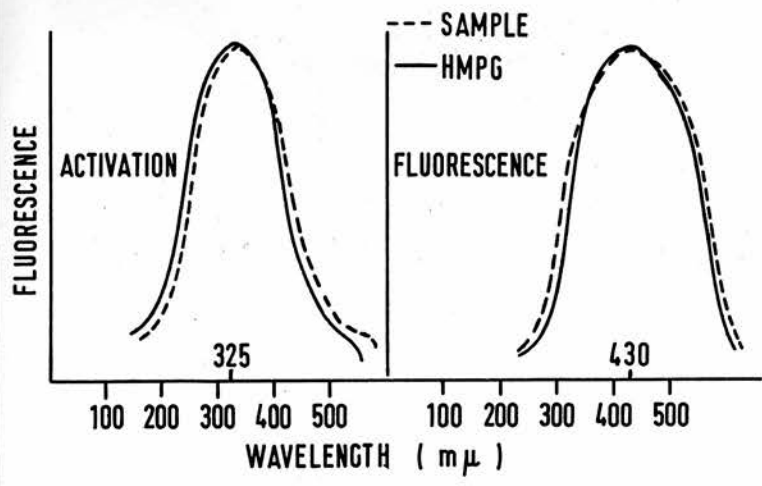


FIGURE 1

FLUORESCENCE MAXIMA - ASSESSED BY ACTIVATION AND FLUORESCENCE SCANS

Figure shows that presumed HMPG in sample and authentic HMPG show maximum fluorescence at identical activation and fluorescence wavelengths.

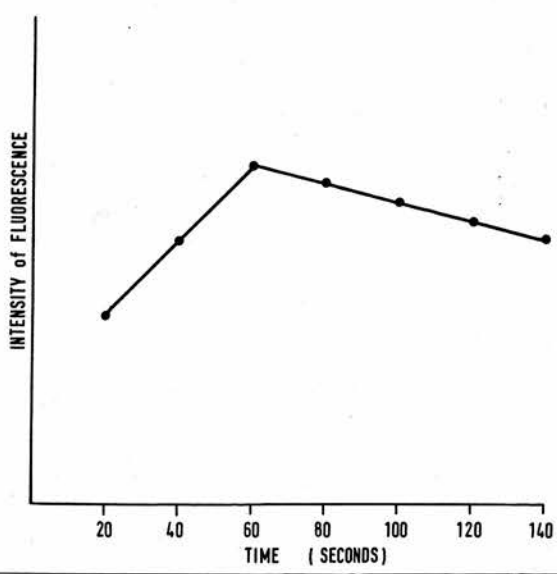


FIGURE 2

EFFECT OF REACTION TIME ON FLUORESCENCE INTENSITY

Figure shows that maximum fluorescence is at 60 seconds. The graph is flatter at >60 <140 seconds and indicates that within this segment fluorescence intensity is more stable.

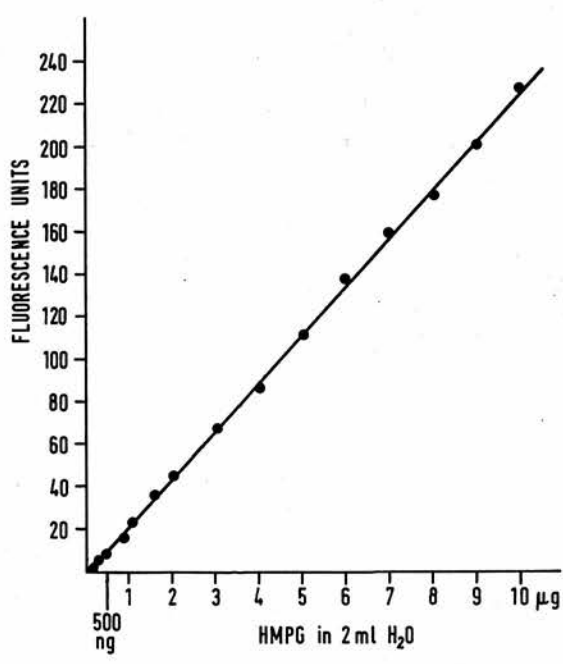


FIGURE 3

LINEARITY OF HMPG FLUORESCENCE

Figure shows that fluorescence intensity of HMPG is linear with concentrations of 50 ng - 10 μg.

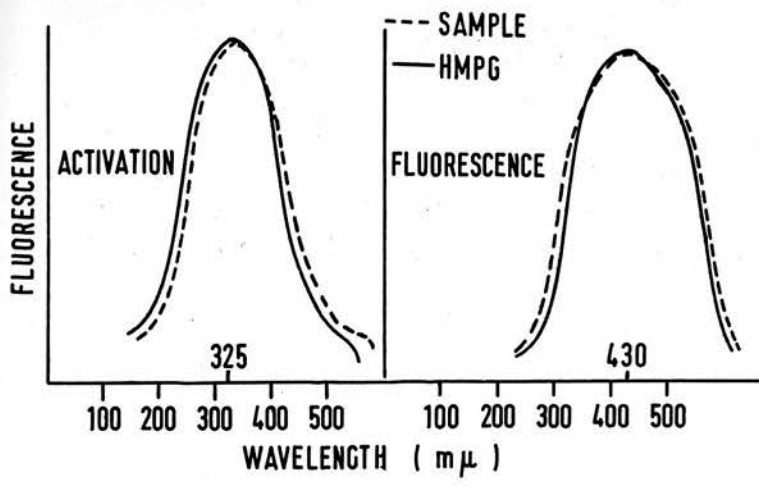


FIGURE 1

FLUORESCENCE MAXIMA - ASSESSED BY ACTIVATION AND FLUORESCENCE SCANS

Figure shows that presumed HMPG in sample and authentic HMPG show maximum fluorescence at identical activation and fluorescence wavelengths.

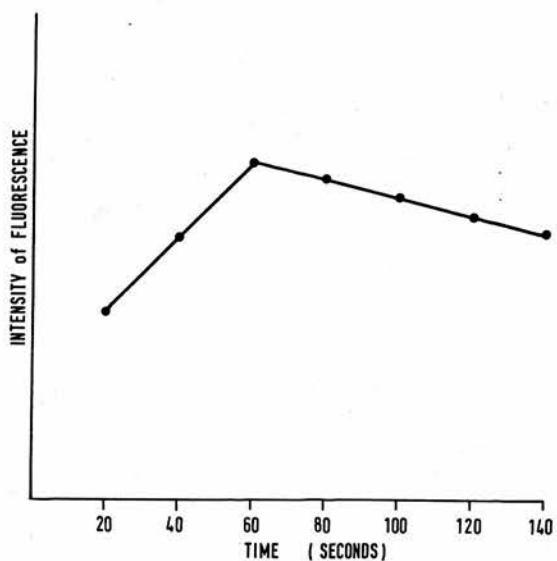


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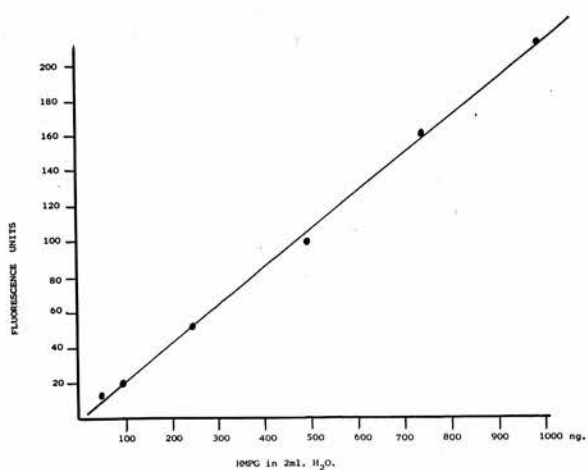


FIGURE 3

LINEARITY OF HMPG FLUORESCENCE

Figure shows that fluorescence intensity of HMPG is linear with concentrations of 50 ng - 10 μg.

500 ng

HMPG in 2 ml H₂O

phenylacetic (HVA) are removed at pH1 in the initial ethyl acetate extraction. The remainder adsorbs onto the resin and is not eluted in the fraction containing HMPG. Of the basic compounds, metanephrine (M), normetanephrine (NM) and 3-methoxytyramine (MTYR), $95 \pm 4\%$ (6) (mean \pm SD (no. of observations)) pass through the column in the effluent and first wash. The remainder stays in the aqueous phase of the second ethyl acetate extraction (pH5-6) to remove HMPG.

The alcohol metabolite of dopamine 4-hydroxy-3-methoxy phenylethanol (HMPT) could still be a contaminant; however, chromatography, *vide infra*, failed to show the presence of this compound.

The method is essentially a determination of the conjugated HMPG in urine. The "free" HMPG was found to be $65 \pm 5\%$ removed by the ethyl acetate extraction at pH1 (for the removal of acids).

(b) FLUOROMETRIC METHOD

The time needed for the ferric chloride reaction to give maximum fluorescence with HMPG was found to be 60 seconds (Figure 2). However, a very minor variation in time produced a sharp change in fluorescence intensity, which could have affected reproducibility. For this reason, 120 seconds was found to be a convenient and optimal time for giving reproducible results; HMPG fluorescence showed a linear curve with concentrations of 100 μg - 10 μg in 2 ml. of distilled water (Figure 3).

Exposure to light caused a deterioration in fluorescence intensity which reverted upon restoring the sample in the dark for one minute.

The reaction was inhibited by ascorbic acid, acetate and phosphate ions; salt quenched the fluorescence.

The limit of sensitivity in the fluorometric procedure, taken as being amount of HMPG giving a fluorescence intensity of twice the blank, was of the order of 50 µg. However, using urine samples the limit of detection was found to be 100 µg/ml.

(c) CHROMATOGRAPHY OF PRESUMED HMPG

In order to validate the identity of the compound in the final extract as HMPG, qualitative thin layer chromatography was performed. The final ethyl acetate extract of the column eluate was blown down to dryness in a stream of nitrogen. The residue was dissolved in 0.2 ml. methanol and applied under a stream of nitrogen to a thin layer plate of cellulose.* Authentic HMPG carrier was added to a urine sample which was processed through the method. The ethyl acetate residue was dissolved in 0.2 ml. methanol which was applied together with authentic HMPG, VMA, NM and M, HVA and HMPT markers to each plate alongside the sample. The plates were developed in one of the following solvent systems:

1. Isobutyl methyl ketone: 4% formic acid (10:1 v/v) - 2 phase (Figure 4).
2. Butanol:ethanol:water (4:1:1 v/v) - single phase (Figure 4a).
3. Butanol:acetic acid:water (4:1:1 v/v) - single phase (Figure 4b).

* TL cellulose precoated plate 20 x 10 cm., 0.1 mm. layer thickness (without fluorescence indicator), E. Merck, A.G. Darmstadt.

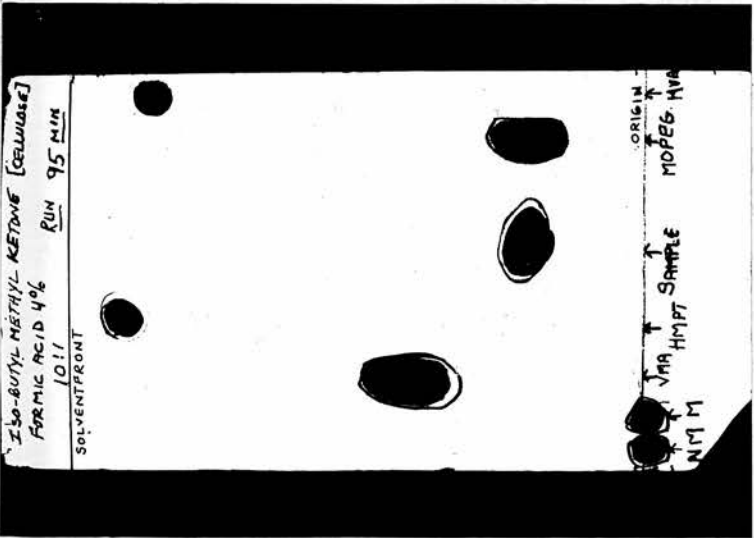


FIGURE 4

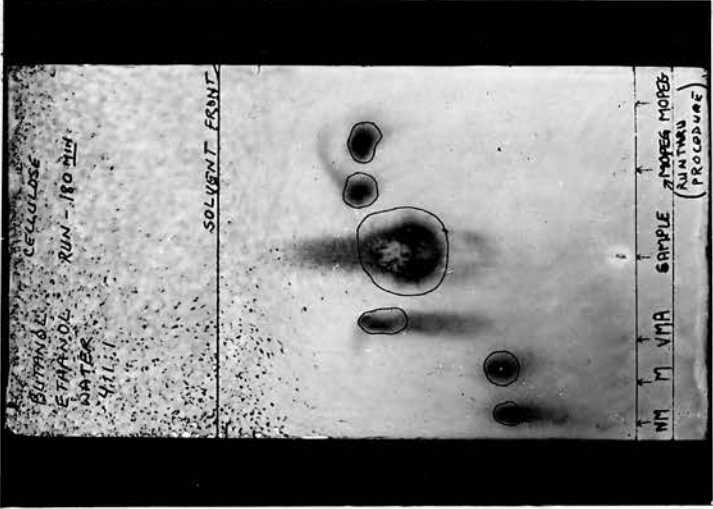


FIGURE 4a

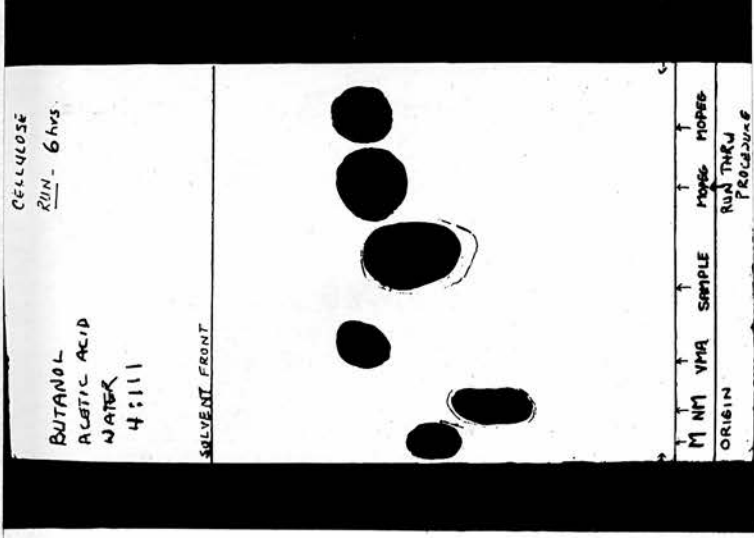


FIGURE 4b

ASCENDING THIN LAYER CHROMATOGRAPHY OF PRESUMED HMPG ISOLATED THROUGH THE METHOD

Cellulose thin layer plates.

Spots visualized by spraying with diazotised paranitroaniline, then ethanolic 1N NaOH.

Spots in Figures 4 and 4b are resotted with china ink prior to photography due to fading of the color.

MOPEG = HMPG



Two phase solvent system: The mixture was shaken and allowed to settle; since the solvents are immiscible in each other, two phases were formed. Most of the organic phase was poured into the chromatography tank and the remainder was used to develop the plate. The aqueous phase was poured into a beaker placed at the bottom of the tank and allowed to flow up a strip of chromatography paper suspended in the tank and its lower edge dipped in the beaker; thus the two phases were kept separate from each other in the tank.

Chromatography technique: The plate was placed in a sealed chromatography tank containing the solvent system and allowed to equilibrate with the solvent's vapours for three hours. It was then developed with an ascending flow until the solvent front reached its upper edge. The plate was removed from the tank, left to dry and sprayed with paranitroaniline (saturated solution in 0.5N HCl) : 10% sodium nitrite in a ratio of 10:1. After drying it was resprayed with 1N NaOH in 50% ethanol. The Rf values of the visualised spots were measured (Table 1).

TABLE 1

THIN LAYER CHROMATOGRAPHY OF PRESUMED HMPG IN SAMPLE

Solvent System	HMPG		VMA		HVA		HMPT		M		NM	
	S*	Mkr.**	S	Mkr.	S	Mkr.	S	Mkr.	S	Mkr.	S	Mkr.
IBMK:formic acid (10:1)	0.29 ⁺	0.29	-	0.48	-	0.84	-	0.81	-	0.00	-	0.00
Butanol:ethanol: water (4:1:1)	0.61	0.63	-	0.64	-	-	-	-	-	0.32	-	0.29
Butanol:acetic acid:water (4:1:1)	0.58	0.59	-	0.66	-	-	-	-	-	0.50	-	0.43

(⁺Rf values: as identified by colour spraying;
S* - sample; Mkr.** - authentic marker)

Comment: In the three solvent systems only one spot with Rf values similar to authentic HMPG was detected.

(d) RECOVERIES THROUGH THE METHOD

Recoveries of 5 μ g HMPG were assessed at the two separate stages of the procedure: (1) column chromatography, (2) solvent extraction.

d.1. COLUMN RECOVERIES: The resin only adsorbs the HMPG loosely (Wilk et al, 1967), retarding its flow through the column. It was found that the volume of the sample:wash:eluate had to be maintained at a constant ratio of 1:2:4. The size of column in relationship to these washes reduced the interference of urinary pigment (which is adsorbed to the column) to a minimum. Thus the optimal column height and diameter for 1:2:3 or 4:5 ml. sample volume were 4 x 0.7: 5 x 0.7: 11 x 0.7 and 7 x 1.2 cm. respectively. In this method quenching from this source amounts to an $8 \pm 2\%$ (4) (mean \pm SD (no. of observations)) decrease in fluorescence intensity. The assessment was done by processing a urine sample through the method and dividing the residue wash (from the eluate ethyl acetate extract) into two equal parts. After adding to one of them 5 μ g of authentic HMPG, their fluorescence intensity was measured. The difference between the two fluorescence intensities was compared with the fluorescences of 5 μ g authentic HMPG in distilled water. The mean column recovery was $84 \pm 6\%$ (9) (mean \pm SD (no. of observations)).

d.2. ETHYL ACETATE EXTRACTION OF COLUMN ELUATE

Effect of salt saturation: Four samples (in duplicate) of 5 μ g HMPG in 12 ml. water were taken; two of them were salt saturated. They

were extracted three times with ethyl acetate (12, 12, 6 ml.). Each pooled ethyl acetate extract was blown down to damp dryness with nitrogen. Each residue was dissolved in 3 ml. water, 2 ml. of which was taken for the fluorometric procedure. Fluorescence intensity of HMPG in the four samples were then compared with each other.

Effect of pH: The following samples (in duplicate) of 5 μ g HMPG in 3 ml. water were adjusted to pH:

1	-	1	sample
2	-	"	"
3	-	"	"
4	-	"	"
5	-	"	"
6	-	"	"
7	-	"	"
8	-	"	"

They were then extracted three times with ethyl acetate (3, 3, 1.5 ml.). Two ml. of each aqueous phase was taken for assessing the fluorescence intensity of HMPG.

Extraction recovery: Three samples (in duplicate) of 5 μ g HMPG in 12 ml. water (pH6-7) were salt saturated and extracted three times with ethyl acetate (12, 12, 6). Each combined ethyl acetate extract was blown down to damp dryness with nitrogen. Each residue was dissolved in 3 ml. water, 2 ml. of which was taken for the fluorescence procedure. The intensity of HMPG fluorescence in each sample (corrected to 3 ml.) was then compared with fluorescence intensity of 5 μ g HMPG in 2 ml. water.

Salt saturation improved the extraction of HMPG into the organic phase by $12 \pm 4\%$ (4) (mean \pm SD (no. of observations)). The HMPG extracted over a wide range of pH from 3-8; below pH3 its extraction

into the organic phase decreased by $10 \pm 3\%$ (4) (mean \pm SD (no. of observations)). Under the conditions of the method, the recovery of this step was found to be $80 \pm 7\%$ (6) (mean \pm SD (no. of observations)).

d.3. HYDROLYSIS: The efficiency of the enzyme hydrolysis, under the same condition used in the procedure, was assessed by the use of Radio Isotopes.

d.3.(i) Materials and Methods

Four male Wistar albino rats weighing 150 gm. each were anaesthetized with ether and each injected intraventricularly (according to the method of Noble et al, 1967) (Appendix II) with 10 μ Ci of DL Normetanephrine* 7-H^3 (3.5Ci/mMol.) in 20 μ l of Merles solution⁽¹⁾. The rats were killed after one hour by cervical fracture, then decapitated. The brains were removed and homogenized, in all glass homogenizers, with chilled 0.4N PCA⁽²⁾ (0.05% ascorbic acid and 0.1% EDTA⁽³⁾) using 4 ml/gm. brain.

The homogenates were spun in a refrigerated centrifuge (4°C) for fifteen minutes at 20,000 g. At this stage 0.2 ml. aliquotes of the supernatants were dissolved in 4 ml. ethanol:methanol (3:1) and 10 ml. of Toluene (0.426% PPO⁽⁴⁾, 0.011% POPOP⁽⁵⁾) scintillator and counted by liquid scintillation, using a Packard (Tri-Carb-Model 3003) liquid scintillation spectrometer to assess the success of the intraventricular injections. Supernatants with $\geq 15,000$ DPM⁽⁶⁾/ml. were pooled and the mixture, which had a pH of 1, was extracted with double volume of ethyl acetate** by shaking for five minutes and

* DL-Normetanephrine 7-H^3 (1mCi in 0.078 mg.), New England Nuclear.
** All reagents used were 'Analar grade' BDH.

centrifuging at 3,000 g for five minutes. After removing the ethyl acetate, the extraction was repeated twice with two further double volumes of ethyl acetate.

90 \pm 5% of the phenolic acids (VMA and HVA) and 65 \pm 5% of the free HMPG were extracted into the organic phase.

The aqueous phase was then adjusted to pH 4 (glass electrode) with 5N and 1N KOH and cooled at 4°C for ten minutes to precipitate the potassium perchlorate. The sample was centrifuged for three minutes at 4,000 g and the supernatant was decanted and adjusted to pH 7.5 (glass electrode) with 1N and 0,1N KOH. Ten ml. of the solution were passed over a 10 x 0.7 cm. O.D. column of amberlite CG50 resin, 100-200 mesh (NH₄⁺ form pH 7.5).

The H³-Normetanephrine together with the other O-methylated amines and 25 \pm 4% (4) (mean \pm SD (no. of observations)) of the free alcohols were adsorbed on to the column. The effluent containing the H³-HMPG sulphate conjugate was collected and concentrated by slow evaporation at 37°C under vacuum.

Abbreviations used:

(1) Merles Solution

NaCl - 8.98 gm.
KCl - 0.25 gm.
CaCl ₂ - 0.14 gm.
MgCl ₂ - 0.11 gm.
NaH ₂ PO ₄ - 0.07 gm.
Urea - 0.13 gm.
Glucose - 0.61 gm.

in 1 lt.
distilled water

- (2) PCA Perchloric acid
- (3) EDTA Ethylene Diamine Tetra acetic acid
- (4) PPO 2,5-Diphenyloxazol
- (5) POPOP 1,4-Dl (2-(5-phenyloxazoly1)) - benzene
- (6) DPM Disintegrations per minute

Preparation of the resin: The resin used was prepared by washing three times with 1N HCl, stirring for half an hour each time. Then it was washed with distilled water until it was free of chloride ions, as tested by the addition of AgNO₃. After that it was washed twice with 3N NH₄OH by stirring for half an hour each time. The NH₄OH was removed by washing with distilled water until the pH fell below 9. The water was decanted and the resin was washed with 0.2M ammonium acetate pH 7.5 until the pH was 7.5-8. The pH was finally adjusted to 7.5 (glass electrode) with glacial acetic acid. At this stage the resin was left overnight and its pH was rechecked and readjusted to 7.5 if needed. Lastly, the ammonium acetate was decanted and the resin was stored in 0.2M ammonium acetate (pH 7.5) ready for use.

d.3.(ii) Purity of presumed H³-HMPG Sulphate Conjugate

Aliquotes of the concentrate were tested for purity of the conjugate by (i) high voltage electrophoresis, and (ii) thin layer chromatography.

In order to test whether H³-Normetanephrine was metabolized to H³-HMPG after its injection, C¹⁴-Noradrenaline was injected into another couple of rats and the C¹⁴-HMPG conjugate was separated. The two labelled conjugates were compared for authenticity and purity.

Preparation of the C¹⁴-HMPG Sulphate Conjugate: Two male Wistar albino rats weighing 150 gm. each were anaesthetized with ether and injected intraventricularly with DL-Noradrenaline* (Carbinol C¹⁴)

* DL-Noradrenaline (Carbinol C¹⁴) DL-bitartrate - The Radiochemical Centre, Amersham.

(25 mCi/mMol.) using 0.5 μ Ci in 20 μ l of Merles solution per rat.

The rats were killed after one hour by cervical fracture, decapitated and their brains removed. The procedure of separating the C^{14} -HMPG conjugate was the same as that used in the separation of the H^3 -HMPG conjugate. Since Noradrenaline was used, the effluent from the CG-50 column contained a mixture of C^{14} -HMPG and DHPG** sulphate conjugates. The effluent from the column was also concentrated under vacuum at 37°C.

High Voltage Electrophoresis: Two aliquotes, 0.2 ml. each, were taken. One consisted of 0.2 ml. of the concentrate containing H^3 -HMPG conjugate and the other a mixture of 0.1 ml. of the concentrate containing H^3 -HMPG conjugate and 0.1 ml. of the concentrated solution containing the C^{14} -HMPG and DHPG conjugates. Each aliquote was applied under a stream of nitrogen onto a strip of 3 mm. Whatman chromatography paper, 47 cm. long and 4 cm. wide. The origins were 7 cm. from the end of the strips on the cathode (negative) side, and the application of each aliquote was confined to an area of 1 x 4 cm.

The strips were electrophoresed at 5,000 volts for 60 minutes in a 6.2M sodium borate buffer (pH 10). They were then dried with nitrogen, cut into 1 x 4 cm. strips and counted by liquid scintillation using 4 ml. of Toluene (PPO, POPOP) scintillator per strip. Blanks were prepared by cutting 1 x 4 cm. strips from a 47 x 4 cm. strip of 3 mm. Whatman chromatography paper which was electrophoresed

** DHPG 3,4-dihydroxyphenylglycol.

in the same buffer system. Each blank strip was immersed in 4 ml. of the same scintillator used for the sample strips. Markers of authentic VMA, HMPG, NM, M and NA* were applied on a separate strip and electrophoresed together with the sample strips. After drying the marker strip was sprayed with a mixture of paranitroaniline and sodium nitrite 10%, allowed to dry and resprayed with ethanolic 1N Na OH. The marker spots were visualized and their distances from the origin were measured.

Results: All samples were kept in the dark for sufficient time to reduce chemiluminescence and temperature adapted before counting, and read against counts obtained from the blank samples. Calculations were done after correction for quenching by the channel ratio method. This method was also used for dual counting (C^{14} and H^3).

At pH 10, HMPG and DHPG conjugates are dissociated since both are acidic by virtue of the strong anionic sulphate (SO_4^{2-}) group on the phenolic ring. The sulphate group substitutes one of the phenolic hydroxyl (OH^-) groups of DHPG and the only hydroxyl group of HMPG (since the second one is already O-methylated). By this DHPG possesses two anionic groups, the sulphate and the hydroxyl, while HMPG has only one, the sulphate group. For this reason, the DHPG conjugate is a stronger acid and thus travelled further than the HMPG conjugate from the origin towards the anode (positive). This resulted in a partial separation of the two conjugates (Figure 5a).

* NA Noradrenaline

The basic compounds (the O-methylated amines) are not dissociated at that pH and, therefore, remained at the origin. At that pH the phenolic acids (HVA and VMA), being stronger acids, travelled further than both conjugates towards the anode. The free alcohols (HMPG and HMPT*) are only weakly acidic by virtue of their phenolic hydroxyl (OH)⁻ group on the 4 position; they travelled only a short distance from the origin towards the anode.

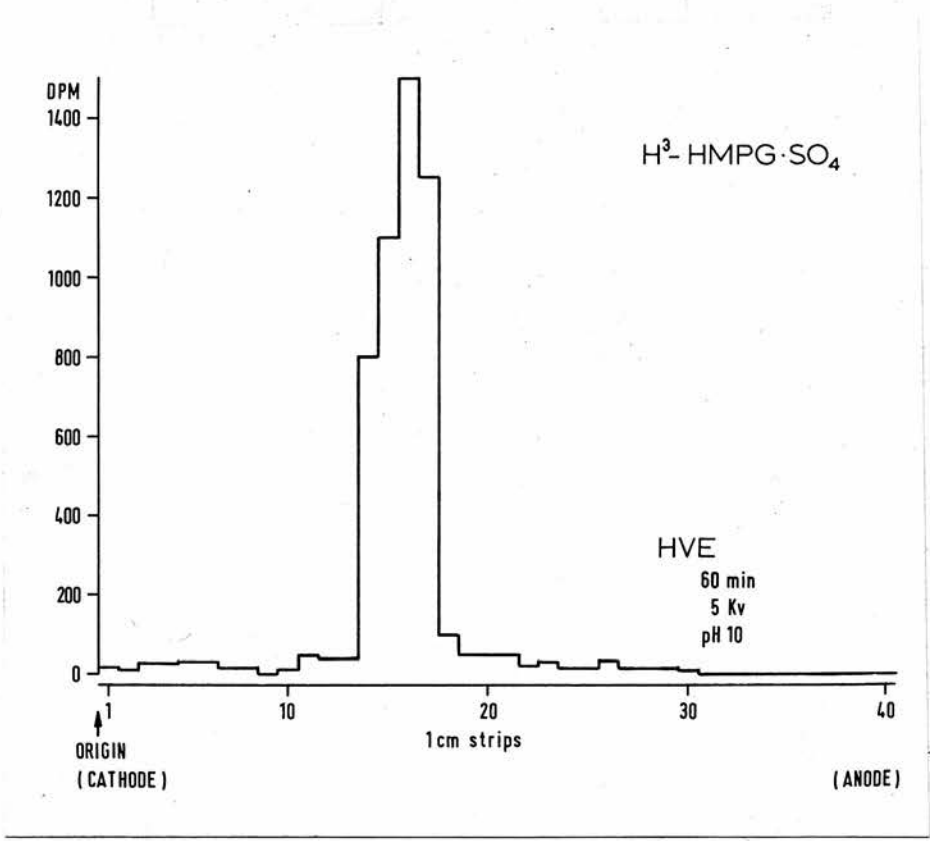
The sequence in which these metabolites travelled from the origin to the anode was: basic compounds (at the origin); free alcohols; HMPG and DHPG conjugates (partially separated) and phenolic acids (nearest the end of the strip on the anode side).

The strips with the H³-HMPG conjugate showed one distinctive peak 16 cm. from the origin (Figure 5) which was assumed to be that of the HMPG conjugate. On the other hand, the strip with mixed H³ and C¹⁴-HMPG conjugates showed two overlapping peaks of C¹⁴ and H³ radioactivity 17 cm. from the origin and another peak of C¹⁴ activity 21 cm. from the origin and only partially separated from the combined peak. This second peak was assumed to be due to the DHPG conjugate (Figure 5a). There were no other peaks of radioactivity along the strips, especially at distances corresponding to NM, NA, HMPG and VMA.

Thin Layer Chromatography: Two aliquotes, 0.2 ml. each, from the concentrate of H³-HMPG conjugate and one 0.2 ml. aliquote from the concentrate containing C¹⁴-HMPG, DHPG conjugates were taken. Each aliquote was made up to 1 ml. with distilled water and adjusted to

* HMPT 4-hydroxy, 3-methoxy phenylethanol.

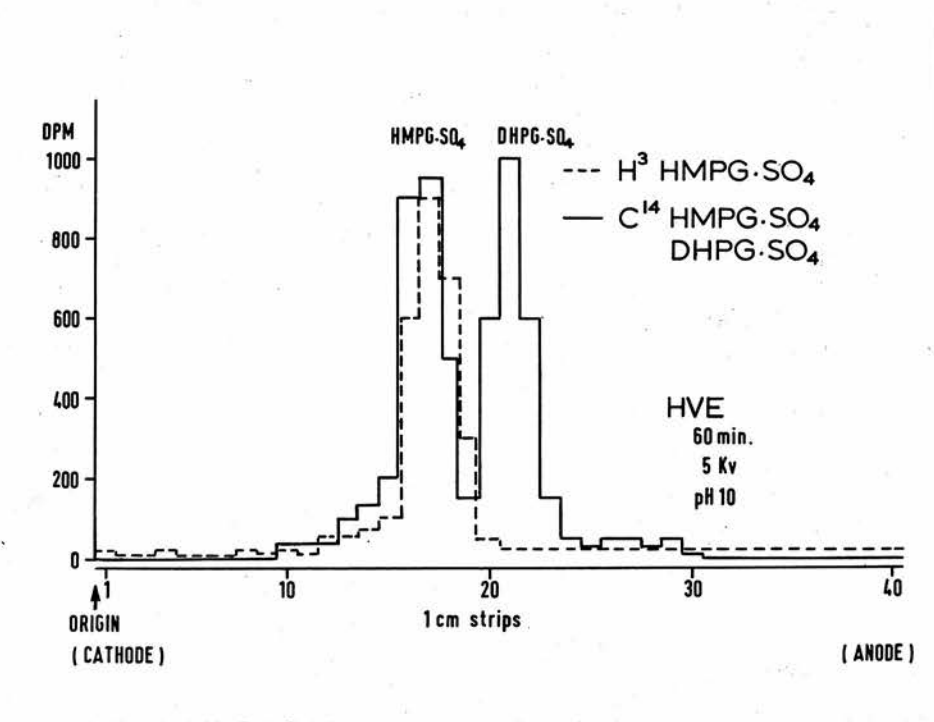
FIGURE 5



PURITY OF PRESUMED HMPG CONJUGATE
(High voltage electrophoresis)

Figure shows that there is one peak of H^3 -radioactivity 16-17 from the origin, presumed to be that of HMPG conjugate. No other peaks are detected.

FIGURE 5a



PURITY OF PRESUMED HMPG CONJUGATE
(High voltage electrophoresis)

Figure shows two overlapping peaks of H^3 and C^{14} - radioactivity 17 cm. from the origin, partially separated from another peak of C^{14} activity. The former peak is presumed to be HMPG conjugate and the latter DHPG conjugate.

pH 5 (glass electrode) with 0.1 ml. 1M sodium acetate buffer (pH 5) and 0.5N HCl. To one of the samples containing the H^3 -HMPG conjugate and the sample containing the C^{14} -HMPG, DHPG conjugates, Helicase enzyme (powder form) was added using 9 mgm. of enzyme powder per sample. The three samples were then incubated in a water bath at $37^{\circ}C$ for 24 hours. At the end of that period the samples were centrifuged for 5 minutes at 3,500 g and the supernatants decanted. The supernatant from each sample was reduced to ≈ 0.4 ml. at $37^{\circ}C$, under vacuum.

From each reduced sample 0.1 ml. was applied, under a stream of nitrogen, onto a cellulose thin layer plate (20 x 10 cm.). The three samples were applied onto three different 2 cm. strips, each of which was delineated on a separate cellulose plate (Appendix IIIa). The strips were delineated all along the length of the plates. On each plate, alongside the strip, markers of authentic HMPG and VMA were applied and onto the plate where the C^{14} -HMPG, DHPG sample was applied, authentic DHPG marker was also used. The plates were developed in Isobutyl methyl ketone: 4% formic acid (10:1^v/v; 2 phase) using the ascending chromatography technique. They were then removed from the tank and dried with nitrogen; the sample strip from each plate was delineated into 1 x 2 cm. portions from the origin to the solvent front. The portions were scraped off the glass plate and suspended into separate scintillation vials containing a mixture of Thixotropic gel* and 10 ml. of Toluene Triton X scintillator (0.4% PPO, 0.01%

* Thixotropic gel Cab-o-cil (finely divided silica)
Reg. Trade Mark of T.M. Godfrey L. Cabot Inc.

POPOP, 50% Triton X 100*). The vials were then counted by liquid scintillation. To visualize the marker spots, the unscraped part of each plate was sprayed with a mixture of paranitroaniline and 10% sodium nitrite, allowed to dry and resprayed with ethanolic 1N NaOH. The Rf values of both HMPG and DHPG markers were calculated.

Results

H³-HMPG Conjugate: The hydrolysed sample showed a major peak of radioactivity at an Rf value identical to that of authentic HMPG. A minor peak at the origin represented unhydrolysed (conjugated) HMPG which, in the solvent system used, does not move from the origin (Figure 6). The unhydrolysed sample showed a single peak of radioactivity at the origin. No other peaks of radioactivity could be detected (Figure 6).

C¹⁴-HMPG, DHPG Conjugates: There were two major peaks of radioactivity corresponding to Rf values of authentic HMPG and DHPG. A minor peak was detected at the origin which probably represented unhydrolysed (conjugates) HMPG and DHPG (Figure 6a).

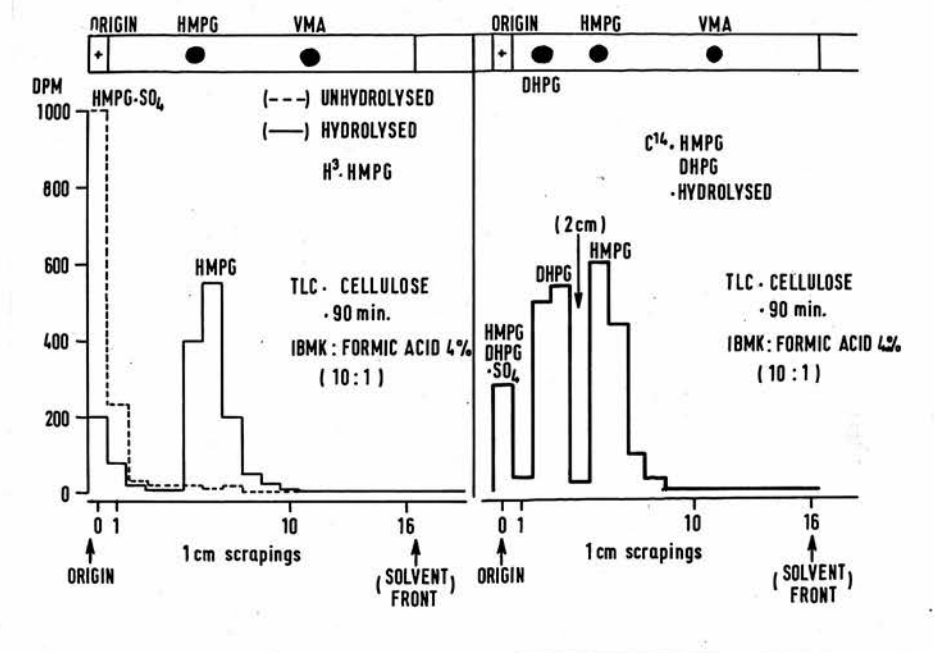
d.3.(iii) Assessment of Percentage Enzyme Hydrolysis in Urine

Hydrolysis Procedure: The remaining concentrated solution of H³-HMPG conjugate was further reduced in volume to \approx 0.6 ml. by slow evaporation at 37°C under vacuum. It was then divided into three equal samples, 0.2 ml. each. To each of the samples 0.8 ml. of unhydrolysed urine and 0.1 ml. of 1M sodium acetate buffer (pH 5) were added.

* Triton X 100 an alkyl phenoxy polyethoxyethanol.

FIGURE 6

FIGURE 6a



PURITY OF PRESUMED CONJUGATE
(Thin layer chromatography)

Figure 6 shows that after enzyme hydrolysis the major peak of radioactivity has an R_f value identical to that of authentic HMPG. The unhydrolysed sample shows one peak at the origin assumed to be HMPG conjugate.

Figure 6a shows that after enzyme hydrolysis there are two peaks of radioactivity at R_f values identical to authentic HMPG and DHPG. A minor peak at the origin probably represents combined HMPG and DHPG conjugates.

Marker spots were visualized by spraying with diazotised paranitroaniline, then ethanolic 1N NaOH.

Nine mgm. of Helicase enzyme (in the powder form) were added to two of the three samples. At that stage 10 μ g of authentic HMPG were added to each sample to protect against degradation of the free HMPG. All samples were adjusted to pH 5 (glass electrode) with 1N and 0.1 N HCl, two drops of chloroform added to each, and were incubated in a water bath at 37°C for 24 hours.

At the end of that period, the samples were centrifuged for 3 minutes at 4,000 g and the supernatants decanted. Each precipitate was then washed with 0.2 ml. of distilled water, centrifuged and the washing added to the respective supernatant. The combined supernatants from each sample were reduced in volume to \approx 0.7 - 0.8 ml. by slow evaporation under vacuum at 37°C.

Separative Procedures

High Voltage Electrophoresis: An 0.2 ml. aliquote (\approx 25,000 DPM) from each concentrated supernatant was applied under a stream of nitrogen onto a 47 x 4 cm. strip of 3 mm. Whatman chromatography paper; the origin was 7 cm. from the end of the strip on the cathode side. The strips were electrophoresed at 5,000 volts for 90 minutes in a buffer system (pH 5.3) of pyridine 25 ml. and acetic acid (glacial) 10 ml. made up to 2.5 lt. with distilled water. Markers of authentic HMPG and VMA were applied on a separate strip which was electrophoresed with the sample strips.

Each sample strip was then dried with nitrogen and cut into 1 x 4 cm. strips, each of which was immersed in 4 ml. of Toluene (PPO, POPOP) scintillator. They were counted by liquid scintillation against 1 x 4 cm. blank strips cut from a strip which was

electrophoresed in the same buffer. The marker strip was sprayed with a mixture of paranitroaniline and 10% sodium nitrite, and the spots visualized by respraying with ethanolic 1N NaOH.

Results: Direct counting of Tritium radioactivity on paper showed that, owing to self-adsorption of the low energy Beta particles and the opacity to light, the efficiency of counting by this method was only 20 per cent.

At an acidic pH of 5.3, basic compounds, like the O-methylated amines are dissociated and travel towards the cathode (negative) side. On the other hand, acidic compounds like the phenolic acids, free and conjugated alcohols are not readily dissociated and, therefore, travel a shorter distance towards the anode (positive) than in an alkaline pH. Depending on their acidic strengths they vary in the degree of dissociation. For that reason the free alcohol (HMPG) with the weakest acidic properties is not dissociated and so remained at the origin. Both the conjugated alcohol (HMPG. SO₄) and the phenolic acid (VMA) travelled towards the anode but, since VMA is the stronger acid, it travelled further towards the anode than HMPG. SO₄ did; thus a separation of 9-10 cm. was obtained (Figure 7).

The duplicate strips on which the hydrolysed aliquotes were applied showed major peaks of radioactivity at the origin and other minor peaks 16-17 cm. from the origin (Figure 7). On the other hand, the strip on which the control (unhydrolysed) aliquote was applied showed one peak of radioactivity 17 cm. from the origin (Figure 7).

Thin Layer Chromatography: Single strips, 2 cm. wide, were delineated along the lengths of three cellulose thin layer plates (20 x 10 cm.).

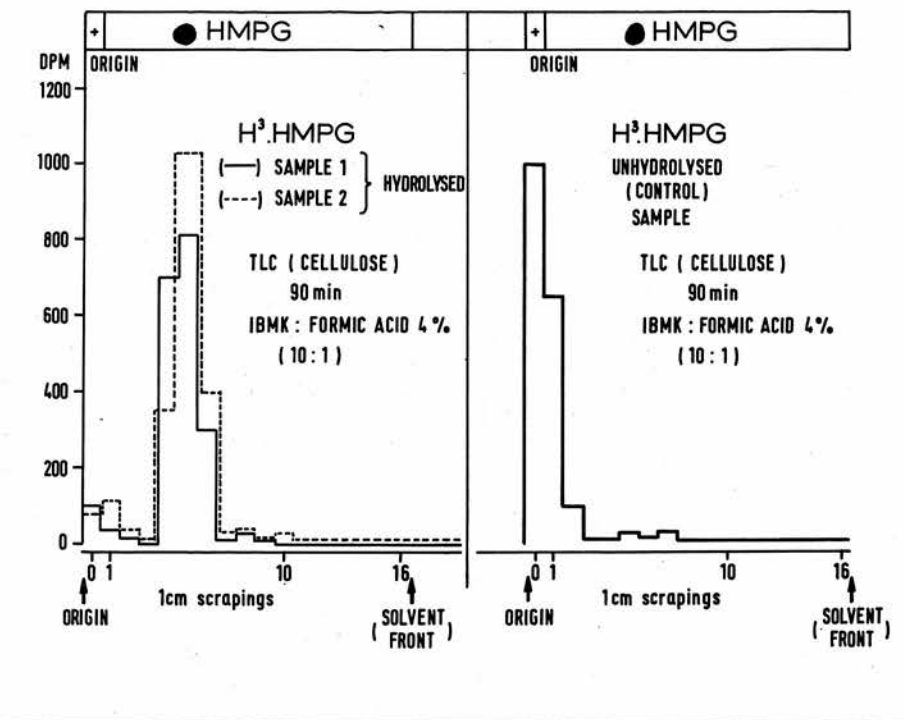
An 0.1 ml. aliquote (\approx 12,000 DPM) from each concentrated supernatant was applied separately onto each strip. Authentic markers of HMPG were applied onto the plates alongside the strips. The plates were chromatogramed in Isobutyl methyl ketone:4% formic acid (10:1 $\frac{v}{v}$), using the ascending technique. They were then removed from the tank and dried with nitrogen. The sample strip from each plate was delineated, from origin to solvent front, into 1 x 2 cm. portions. Each portion was separately scraped off the glass plate, suspended in Thixotropic gel and 10 ml. of Toluene Triton X (PPO, POPOP) scintillator and counted by liquid scintillation against blanks obtained from scrapings off a plate developed in the same solvent system. The unscraped part of each plate was sprayed with a mixture of paranitroaniline and sodium nitrite, left to dry and resprayed with ethanolic 1N NaOH. The Rf values of the visualized HMPG marker spots were calculated.

Results: The duplicate hydrolysed samples showed major peaks of radioactivity at Rf values identical to those of authentic HMPG (Figure 8). Minor peaks at the origin were due to the unhydrolysed (conjugated) HMPG which, in the solvent system used, remains at the origin. The control, unhydrolysed sample showed one peak of radioactivity at the origin (Figure 8a).

Column Chromatography: Two 0.2 ml. aliquotes (\approx 25,000 DPM) were taken from each of the three concentrated supernatants. One of the two aliquotes from each supernatant was dissolved in 10 ml. of Toluene Triton X (PPO, POPOP) scintillator and counted by liquid scintillation, thus giving the total radioactivity of both free and

FIGURE 8

FIGURE 8a



**ASSESSMENT OF PERCENTAGE HYDROLYSIS
IN URINE BY THIN LAYER CHROMATOGRAPHY**

Figure 8 shows that the duplicate hydrolysed samples have a major peak of radioactivity at an Rf value identical to authentic HMPG.

Figure 8a shows that the unhydrolysed sample has only one peak at the origin, presumed to be HMPG conjugate, which does not travel in the solvent system used.

Marker spots were visualized by spraying with diazotised paranitroaniline, then ethanolic 1N NaOH.

conjugated HMPG in the aliquote of each concentrated supernatant. Each of the other 0.2 ml. aliquotes was made up to 1 ml. with distilled water and passed over a column (4 x 0.7 cm. OD) of untreated Bio-Rad AGI-X4 resin. Each column was then washed with 2 ml. of distilled water and the HMPG was eluted off the column with 6 ml. of distilled water. A 1 ml. aliquote from each eluate was dissolved in 10 ml. of Toluene Triton X (PPO, POPOP) scintillator and counted by liquid scintillation.

Results: The HMPG conjugate adsorbs onto the column and partially elutes off with a strong acid (i.e. 6N acetic acid) and not water which elutes off 80 per cent of the free HMPG.

The 1 ml. aliquote from the eluates of the duplicate hydrolysed samples showed high levels of radioactivity while that from the eluate of the control (unhydrolysed) sample had a blank (background) level of radioactivity.

Results of Percentage Hydrolysis

All counts of radioactivity were corrected for quenching by the channel ratio method, thus values were expressed as DPM. Assessment of percentage hydrolysis, using high voltage electrophoresis and thin layer chromatography, was obtained by adding the total radioactivity representing both free and conjugated HMPG and calculating the percentage of each from values obtained from the individual peaks separately (hydrolysed HMPG and conjugated HMPG). Percentage hydrolysis, using column chromatography, was calculated from the ratio of:

$$\frac{\text{Radioactivity (DPM) in the eluate from hydrolysed sample}}{\text{Total radioactivity (DPM) in 0.2 ml. aliquote of supernatant.}}$$

TABLE 2

RESULTS OF PERCENTAGE HYDROLYSIS

EXP	HVE		TLC		C.C.	
	S1*	S2**	S1	S2	S1	S2
1	78	80	75	76	80	82
2	82	81	80	77	84	80

* S1 - Sample 1
** S2 - Sample 2

HVE - High voltage electrophoresis
TLC - Thin layer chromatography
C.C.- Column chromatography

Comment: From the above figures the efficiency of enzymatic hydrolysis appeared to be fairly uniform with a mean of: $79.58 \pm 2.6\%$ (12) (mean \pm SD (no. of observations)).

(e) TOTAL RECOVERY

This was assessed by processing, in triplicate, eight different samples of urine, to each of which had been added $5 \mu\text{g}$ HMPG prior to the enzyme hydrolysis step. The recoveries from each sample are given in Table 3.

TABLE 3

TOTAL RECOVERY OF HMPG THROUGH THE METHOD

Urine Sample No.	% Recovery of HMPG
1	55 \pm 2 (3)*
2	57 \pm 3 (3)
3	59 \pm 3 (3)
4	61 \pm 3 (3)
5	60 \pm 3 (3)
6	56 \pm 4 (3)
7	63 \pm 2 (3)
8	60 \pm 2 (3)

* Mean value \pm SD (no. of estimates)

Total recovery = 59 \pm 4% (24) (mean \pm SD (no. of observations))

(f) LEVELS OF HMPG IN URINE OF NORMALS

Urine was collected over a 24 hour period from five male and seven female subjects (age range 25-50 years) who were on a diet which reduced the intake of sympathomimetic compounds, amines and their precursors. The urine was preserved with 10 ml. 6N HCl at 4°C until assay, which was carried out within 24 hours after the collection. The results for the concentration of HMPG in these samples are shown in Table 4.

TABLE 4HMPG EXCRETION IN NORMALS(HMPG in $\mu\text{g}/24$ hour urine)

	Males	Females
1	518.67	1508.24
2	1224.78	1171.65
3	938.54	670.41
4	1240.65	313.63
5	696.67	129.31
6		132.10
7		530.91

Mean (male and female) 756.29 ± 459.46 (12)
 Mean \pm SD (no. of observations)

The t-test for uncorrelated means (Guilford, 1956) showed that
 t (d.f. 10) = 1.078 ($t > 2.228$, significant at 0.05 level).

The HMPG values ($756.29 \pm 459.46 \mu\text{g}/24$ hours) found in normals using this method fall within the range published by Wilk et al (1967) (0.25 - 1.49 $\mu\text{g}/\text{mgm. creatinine}$ - mean 0.86 ± 0.5 SD) and Maas et al (1968) (1660 - 892 $\mu\text{g}/24$ hours). No statistically significant sex difference was found (M - 923.86 ± 318.96 SD; F - 636.61 ± 528.3 SD) although (i) the number of subjects was small, and (ii) the values fall over a wide range. This is important because Maas et al (1968) have reported such a difference when HMPG values were expressed as $\mu\text{g}/24$ hours.

*diff. between
 W & F no. are*

(2) VMA (3-methoxy, 4-hydroxy mandelic acid)

The 24 hour urinary VMA levels were determined using the method of Pisano et al (1962). The pooled ethyl acetate containing VMA (extracted from 5 ml. urine aliquote, vide supra - HMPG method) was extracted with K_2CO_3 buffer; VMA in the buffer layer was then converted to vanillin. The latter was extracted into Toluene and back extracted into K_2CO_3 ; its concentrations were measured by spectrophotometry. The sensitivity of the method was adequate for assessing any change in urinary levels of VMA. Levels of VMA in the aliquotes' extracts were within the sensitivity range of the method. Recoveries of VMA by the method were uniform with a mean value of $84 \pm 8\%$ (36) (mean \pm SD (no. of observations)).

APPLICATION OF BIOCHEMICAL METHODS

(A) CHRONIC SCHIZOPHRENIC PATIENTS (RECEIVING L-METHIONINE)

(1) SELECTION OF PATIENTS: Six male patients, comprising the third group transferred to the metabolic ward, were studied.

(2) URINE COLLECTION: Urine was collected from each patient for 24 hours, every other day throughout the twelve week period. The urine was put in bottles containing 10 ml. (6N) HCl and stored at 4°C. The collection was under close surveillance by the nursing staff and during the 24 hours of collection the patients were confined to the ward. The door of the metabolic toilet was provided with a warning bell so as to ensure complete collection.

(3) DIET: Throughout the twelve week period the six patients received a standardized diet which was reduced in items containing "sympathomimetic compounds" (i.e. Tyramine) and amines or their precursors. Items excluded were: yoghurt, fava beans, cheese, wine, citrus fruits and juices, cola beverages, bananas, chocolate and cocoa, coffee, tea and vanilla. Fluid intake was not restricted but a fluid balance sheet (intake - output) was charted for each patient throughout the twelve week interval.

(4) ESTIMATION OF VMA AND HMPG: All urine samples were analysed fresh after collection. The volume of each sample was measured and an aliquote of 5 ml. was taken. The 24 hour urinary levels were calculated from the values of each in the aliquote and the 24 hour urine volume. The values were expressed as $\mu\text{g}/24$ hours.

*Test. urine
aliquots by
or aliquots*

(B) ACUTE SCHIZOPHRENIC PATIENTS

(1) SELECTION OF PATIENTS: The criteria for selection were age, sex and treatment. Five acute male admissions, ages ranging between 25-60 years, were included. None had received any drugs (phenothiazines, butyrenphenones or barbiturates) for at least six weeks prior to admission. As each patient was admitted he was transferred to the metabolic ward for a "drug free" period of three days. The diagnosis of acute or subacute schizophrenia was made by the admitting psychiatrist and confirmed by the investigator (F.A.). All the patients were free from physical or other psychiatric illnesses.

(2) URINE COLLECTION: During the period of stay in the metabolic unit, three 24 hour urine collections were taken under the same close surveillance obtaining for the schizophrenic patients. The urine was collected in plastic bottles containing 10 ml. (6N) HCl and stored at -20°C until the last sample was collected three days later. All three samples were assayed at the same time.

(3) DIET: An identical diet as was used for the chronic schizophrenics was given to the patients during urine collection and for one day prior.

(4) ESTIMATION OF HMPG: The three urine samples were defrosted and their volumes measured. A 5 ml. aliquote was taken from each sample for the determination of HMPG which was expressed as $\mu\text{g}/24$ hours.

(C) NORMAL CONTROLS

(1) SELECTION OF SUBJECTS (7 females and 5 males): Twelve normal volunteers, free from mental and/or physical ailments, ages ranging between 25-50 years, were selected. 7/12/50

(2) URINE COLLECTION: Three 24 hour samples of urine were collected from each subject on alternate days and stored in plastic bottles with 10 ml. (6N) HCl at 4°C until assay. The volunteers were informed about the importance of complete collection.

(3) DIET: The same diet used for the schizophrenic population was given to the controls for a period of eight days, including a 24 hour interval prior to urine collection.

(4) ESTIMATION OF HMPG: The 24 hour urine samples were measured and assayed for HMPG in one batch within 24 hours of collection. An aliquote of 5 ml. was taken from each sample for the determination of HMPG; the values were expressed as $\mu\text{g}/24$ hours. The HMPG results from both groups were used to assess the reliability of the HMPG method, vide supra, and the HMPG levels from the male group were used for comparison with the HMPG levels from the chronic and acute schizophrenic patients.

R E S U L T S

(A) CLINICAL

Before the start of the experiment, two out of the five patients in the pilot study and seven out of the eleven patients in the main experiment were receiving psychotropic drugs (i.e. phenothiazines and butyraphenones). None of these nine patients showed any worsening of their schizophrenic symptoms when these were stopped. On the contrary, one patient (Appendix I (b) 1) showed a minor improvement in her mental state.

(1) PILOT STUDY: Of the five patients, two showed a reaction to L-methionine, becoming deluded and exhibiting thought content and form disorders (Appendix I (a) 1,2). The other three did not show any changes in their clinical states on either L-methionine or glycine (Appendix I (a) 3,4,5). In neither group was there any clouding of consciousness or signs of vestibular and/or cerebellar dysfunction. The "methionine reaction" became less acute after two days and persisted until the amino acid was withdrawn. In both affected patients a remission to the pretreatment mental state followed the discontinuation of L-methionine. None of the five patients showed any clinical improvement after L-methionine. Glycine proved inert.

On the Lorr rating scale (two independent raters) similar patterns of symptom fluctuations were apparent (Figure 9 - 1,2). The symptom ratings on the scale were generally in agreement with the finding from the psychiatric interview (Figure 9 - 1,2,3,4,5).

1 ——— L-Methionine ——— Glycine ———

2 ——— Glycine ——— L-Methionine ———

3 ——— L-Methionine ——— Glycine ———

4 ——— L-Methionine ——— Glycine ———

5 ——— Glycine ——— L-Methionine ———

Henry Phipps
Lorr Scale
65
63

Amino Acid
A

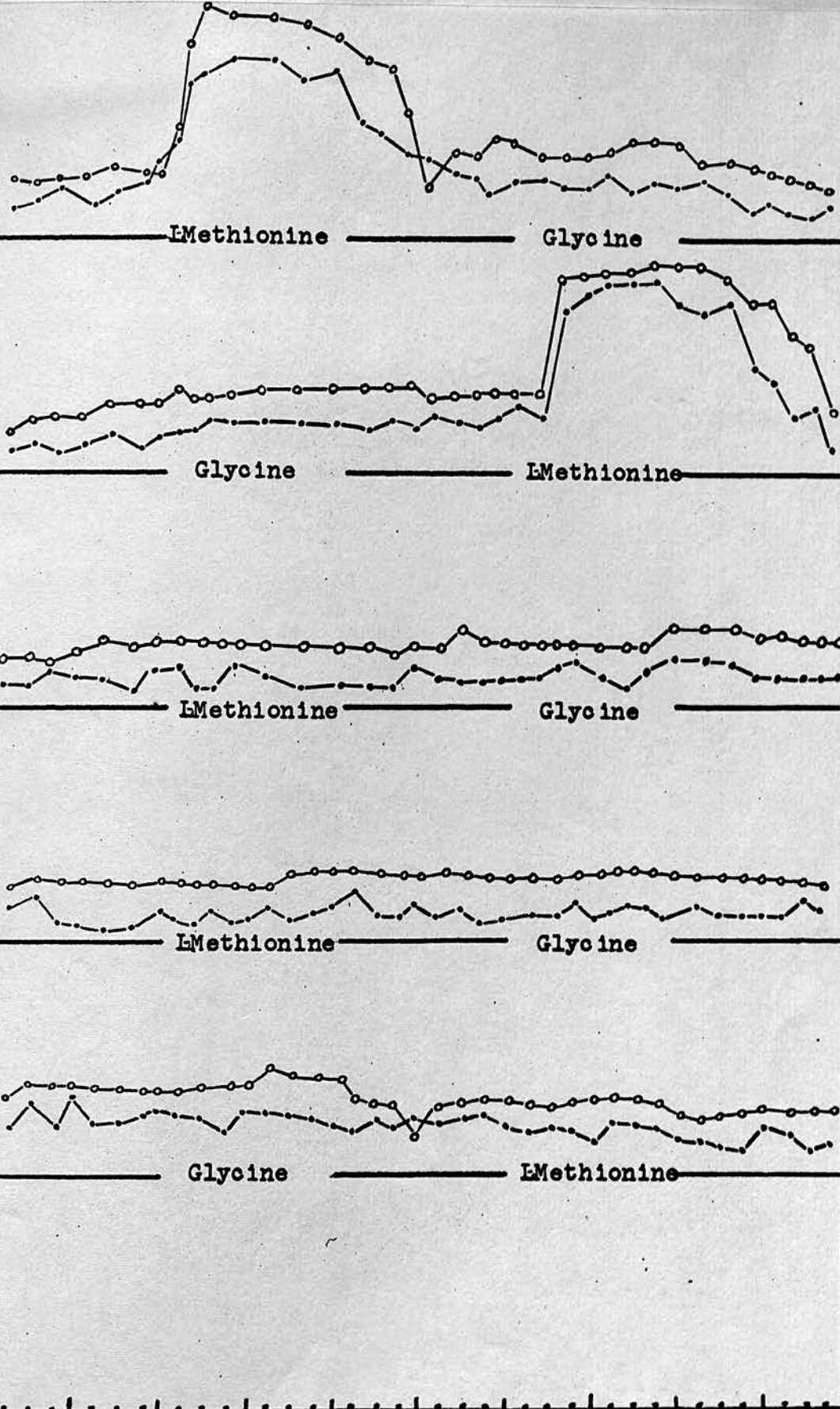
Amino Acid
B

• Rating day
± 1 Week period
+ 2 Week period

PILOT EXPERIMENT
(Females)

--- Lorr Scale
—○— Henry Phipps

FIGURE 9



The Henry Phipps nurses' rating scale and observations from the patients' ward behaviour showed clinical changes in the two patients which were in broad agreement with the changes observed by the rating physicians (Figure 9 - 1,2).

Both the medical and nursing staffs were in accord that the L-methionine reaction was schizophreniform in nature. Neither group could detect any signs or symptoms indicating a "toxic" delirium.

(2) MAIN EXPERIMENT: Of the eleven patients (five female and six male), seven showed a clearcut reaction to L-methionine, and four were not affected. None of the patients reacted to glycine. All the eleven patients tolerated both amino acids well physically and none showed gastrointestinal disturbance.

In two of the seven L-methionine reactors (one female and the other male), the amino acid "B" (found later to be L-methionine) had to be withdrawn owing to the marked psychiatric symptoms. In the case of the female patient (Appendix I (b) 1) L-methionine was discontinued after seven days. After an interval of ten days (day seventeen), phenothiazines were started, to which she responded favourably. In the male patient (Appendix I (b) 7) L-methionine was stopped after eight days; he also was given phenothiazines on the fourteenth day and responded well to them. In both patients there was an initial reaction to L-methionine, apparent after the first day, which lasted for 24-48 hours. This was toxic in nature with vestibular signs and ataxia, coupled with disordered sensorium and orientation. After the reaction subsided, a twelve hour period

of remission followed before another reaction of a different nature appeared. This latter, which developed in all seven patients about three to four days after the start of L-methionine, was characterized by the absence of any such disturbances in the levels of consciousness, or other toxic signs, and was schizophreniform in nature (Appendix I (b) 1,2,5,6,7,10,11). In five reactors (Appendix I (b) 1,2,7,10,11) there was a worsening of the existing symptoms, some of which became more frequent and florid. In three reactors, quiescent schizophrenic symptomatology (which the patients showed during the earlier stages of their schizophrenia) returned and became clinically obvious. Three of the patients who reacted to L-methionine had a recurrence of specific elements of thought content disorder that they had shown years before (Appendix I (b) 1,2,11).

In all seven patients the schizophreniform psychoses subsided on withdrawing L-methionine. Five of these patients were started on phenothiazines (Appendix I (b) 1,2,6,7,10) and two were not (Appendix I (b) 5,11). Only three of the seven reactors (Appendix I (b) 1,2,6) showed minor improvement that was characterized by "greater spontaneity" and more social interaction. In one the improvement was short-lived, whilst in the other two patients (2,6) it was sustained. None of the group who reacted to L-methionine became worse after its withdrawal, and they all reverted to their pre-methionine mental states. None of the reactors not requiring drugs before the experiment needed them at the end. In the remaining four patients, no significant clinical changes were

noticed during the entire experiment (Appendix I (b) 3,4,8,9), although one of these did become slightly better (Appendix I (b) 3). At follow-up, one year later, all except one patient (Appendix I (b) 8), who died from an unrelated physical disease, were essentially the same mentally as at the pre-experimental assessment.

The two rating psychiatrists were in agreement about the development and nature of the symptoms. Their conclusions, based on the Lorr scale scores and psychiatric interviews, were similar to, and complemented, the findings obtained from the Henry Phipps' scores and ward behaviour observations (Figure 10, 1-11). The latter helped the psychiatrists to probe for symptoms and signs which otherwise might have been overlooked.

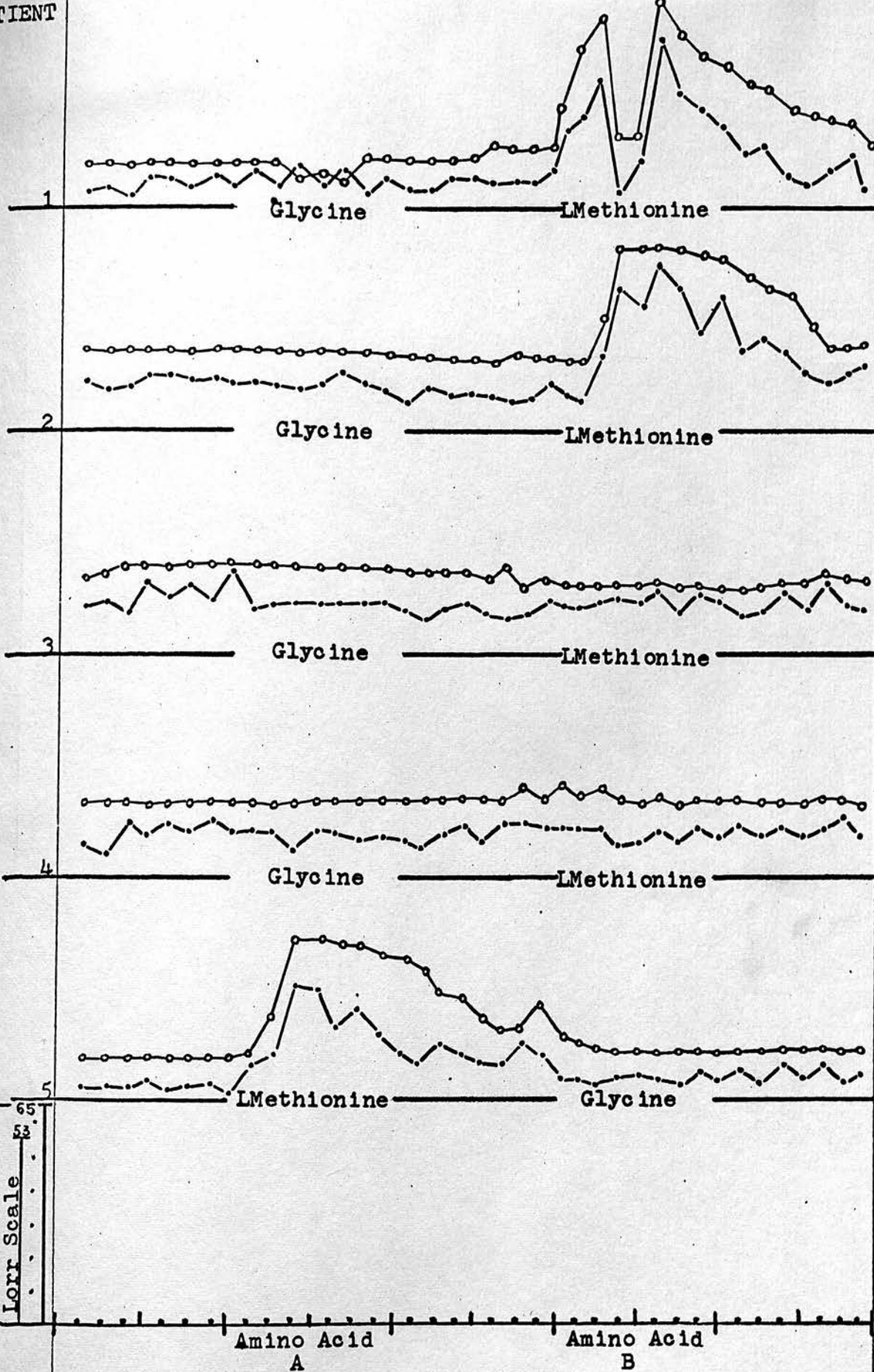


FIGURE 10

Henry Phipps

Lorr Scale

• Rating day
 ⊥ 1 Week period
 + 2 Week period

MAIN EXPERIMENT
 (Females)

—•— Lorr Scale
 ○—○ Henry Phipps

Amino Acid A

Amino Acid B

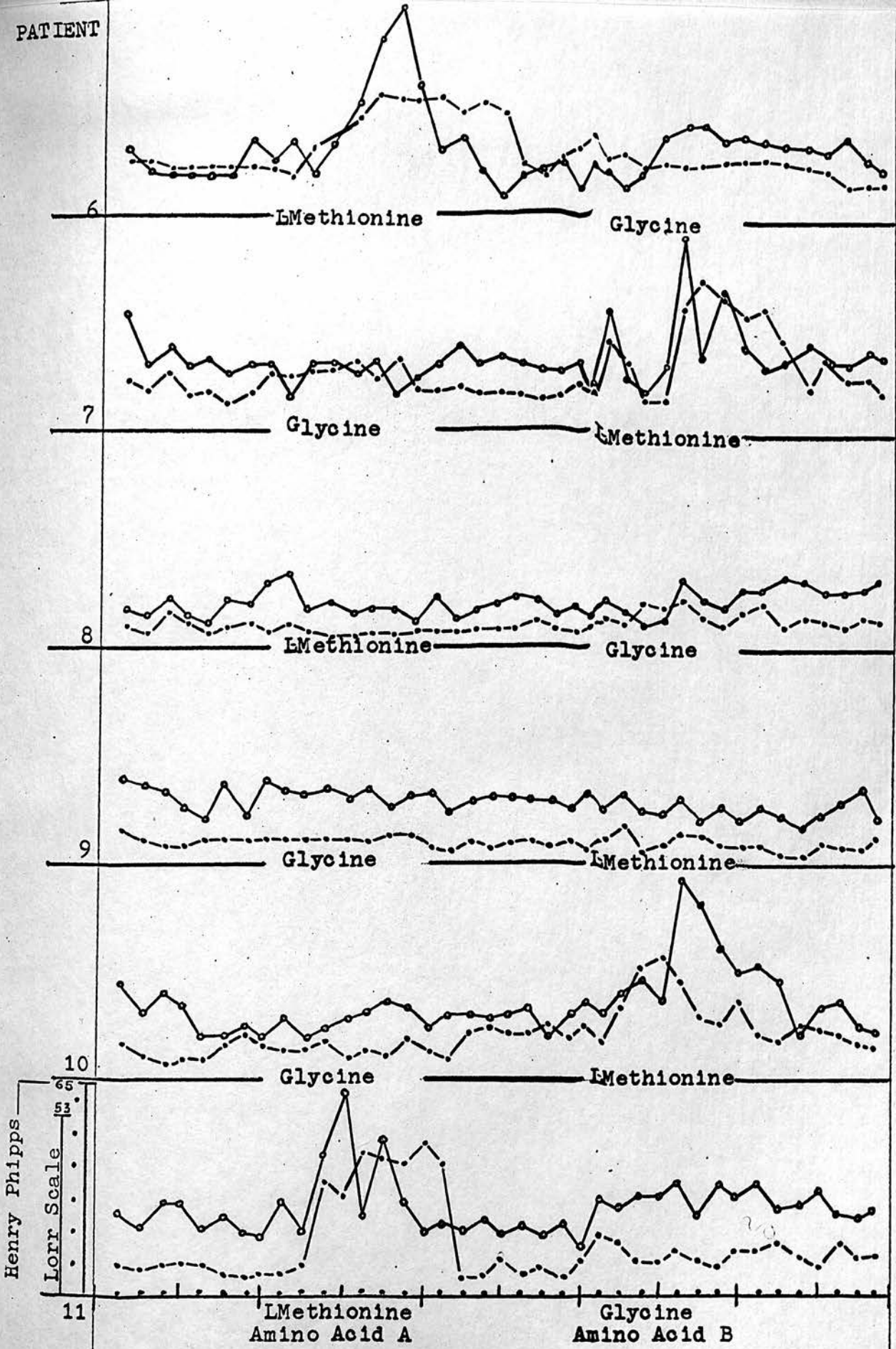


FIGURE 10 (Contd.)

Henry Phipps

Lorr Scale

• Rating day
 + 1 Week period
 + 2 Week period

MAIN EXPERIMENT
 (Males)

--- Lorr Scale
 -o- Henry Phipps

TABLE 5
SUMMARY OF CLINICAL RESULTS

Total	Main Experiment	Pilot	Sex	Number	Reactor	Non-Reactor	Treatment before trial	No treatment before trial	Treatment after trial	No treatment after trial	No change	Better	Worse	Reaction			
														Toxic	Same Symptoms Worse	Quiescent Symptoms Reappear	
	F	F	F	1	+		+			+	+				+		
	F	F	F	2	+		+	+		+	+				+		+
	F	F	F	3		+		+		+	+						
	F	F	F	4		+		+		+	+						
	F	F	F	5	+	+	+			+	+						
	M	F	F	6	+		+		+			+					
	M	F	F	7	+		+		+			+		+			
	M	F	F	8		+	+		+			+					
	M	F	F	9		+	+		+			+					
	M	F	F	10	+		+		+			+				+	
	M	F	F	11	+			+		+		+				+	+
16					9	7	9	7	6	10	12	4	0	2	7	4	

(B) BIOCHEMICAL

(1) CHRONIC SCHIZOPHRENICS

(i) VMA Excretion: The 24 hour urinary VMA levels in each of the six male patients showed wide fluctuations over the twelve week period (Appendix IV). The Kruskal Wallis one-way analysis of variance (Siegel, 1956) showed that VMA mean values increased after the Nil 1 condition (the four week observation before amino acid A); (Tables 6 and 6a). There was no sharp increase in the VMA 24 hour urinary levels during L-methionine feeding.

TABLE 6

VMA EXCRETION

(Mean values of VMA as $\mu\text{g}/24$ hour urine)

Patient	Nil 1 (Prior to Drug A)	Nil 2 (After Glycine)	Nil 3 (After Methionine)	Glycine	Methionine
1 ⁺	1777.3	2788.1	2427.4	2895.1	2242.0
2 ⁺	2236.5	2885.3	3346.8	2805.0	3401.1
3	988.2	3185.9	1798.7	2613.5	1865.8
4	2025.0	2191.6	3940.6	3193.3	3091.4
5 ⁺	1289.4	2947.7	4927.7	2539.2	3885.9
6 ⁺	1803.7	3451.6	2440.5	3199.5	2626.1

No. 1-6 = 6-11 in Table 5 and Appendix I (b)

⁺ The patient reacted to L-methionine clinically.

TABLE 6aKRUSKAL WALLIS (ONE-WAY ANALYSIS OF VARIANCE)

	Nil 3	Nil 2	Glycine	Methionine	Nil 1
Nil 3	-	-	1	5	84 **
Nil 2	-	-	1	5	84 **
Glycine	-	-	-	4	83 **
Methionine	-	-	-	-	79 *
Nil 1	-	-	-	-	-

* $p < 0.06$ ** $p < 0.05$

The tables show that VMA excretion increased after the Nil 1 condition but showed no significant increase on L-methionine loading.

(ii) HMPG Excretion: The 24 hour urinary HMPG levels varied less over the twelve week period, within each patient, than those of VMA (Appendix IV). In one reactor (Appendix I (b) 10) the mean values of 24 hour urinary HMPG from each two week period showed a tendency to increase after the Nil 1 interval (four weeks prior to amino acid A); this increase was about threefold during L-methionine feeding (Table 7, no. 5). However, as a group there was no statistically significant increase in the 24 hour urinary excretion of HMPG on L-methionine loading (two-way analysis of variance - Scheffé, 1959) (Table 7).

TABLE 7

HMPG EXCRETION

(Mean values of HMPG as $\mu\text{g}/24$ hour urine)

Patient	Nil 1 (Prior to Drug A)	*Nil 2	Glycine	Methionine	TR***
1 ⁺	1665	1319	1007	1959	5950
2 ⁺	1516	1917	1718	1372	6523
3	1381	1478	1232	1878	5969
4	1694	1017	1557	700	4968
5 ⁺	1784	2825	2245	6581	13435
6 ⁺	1327	1591	1048	1514	5480
TC**	9367	10147	8807	14004	

No. 1-6 = 6-11 in Table 5 and Appendix I (b).

⁺ The patient reacted to L-methionine clinically.

* Nil 2 - After L-methionine and glycine, values of both periods were combined and the mean value calculated.

** TC - Total columns

*** TR - Total rows

Two-way analysis of variance gave an F-value of 1.03 ($F > 3.29$, significant at 5%); thus there was no increase of HMPG urinary levels on L-methionine loading.

(2) ACUTE SCHIZOPHRENICS - Normal Controls

HMPG Excretion: A one-way analysis of variance (Bliss, 1967) was done to see if there was a statistically significant difference in the levels of 24 hour urinary HMPG among the acute schizophrenics, normal controls and chronic schizophrenics. The F-value was 7.15 (F >6.7; significant at 1% level) (Table 8).

TABLE 8

HMPG EXCRETION IN 24 HOUR URINE OF SCHIZOPHRENICS AND NORMALS

No.	Chronic Schizophrenics	Acute Schizophrenics	Normal Controls	
1	3358.75*	1977.4**	518.67**	
2	1370.00	5820.15	1224.78	
3	1492.25	1855.82	938.57	
4	1630.75	3485.00	1270.65	
5	1242.00	3650.00	696.67	
6	1487.5			
C	10581.25	16788.37	4649.34	G=32018.96

* Mean value of HMPG ($\mu\text{g}/24$ hours) during twelve weeks

** Mean value of HMPG ($\mu\text{g}/24$ hours)

C - Sum of column; G - Grand total of $C_1 + C_2 + C_3$

To determine the location of the difference, Tukey's test for all pairwise differences was used. The studentized range (q_{vr}), as tabulated by Pearson and Hartley (1966), and the calculated values were as follows:-

$$q_{vr}^1 \text{ (chronic-acute) } = 3.60 \text{ (} q_{vr} > 3.18 \text{ - significant at 10\%)}$$

$$q_{vr}^2 \text{ (chronic-normal) } = 1.88$$

$$q_{vr}^3 \text{ (acute-normal) } = 5.25 \text{ (} q_{vr} > 4.96 \text{ - significant at 1\%)}$$

As seen from the results there were significant differences in the levels of 24 hour urinary HMPG between (i) the acute and chronic schizophrenic groups, and (ii) the acute schizophrenic and normal control groups; the greater difference being between the latter two groups. No significant difference was found between the chronic schizophrenic and normal control group.

D I S C U S S I O N

(A) CLINICAL

The present findings, in broad agreement with those of previous workers, have shown that chronic schizophrenic patients tend to fall into two populations - L-methionine "reactors" and "non-reactors"; there was no obvious clinical difference between these two groups. That is, it was not possible clinically to predict whether any particular patient would react or not. It was felt that the reaction observed was most likely due to L-methionine rather than a relapse secondary to phenothiazine withdrawal because (i) three reactors had not received any medication for four to five years prior to the experiment, and (ii) some of the non-reactors did not show any relapse on withdrawal of the phenothiazines. It could be argued that L-methionine produced a non-specific stress reaction which might have triggered a schizophrenic relapse, especially in patients unprotected by psychotropic drugs. Such stress phenomena are known to contribute to schizophrenic relapses but, interestingly enough, of all the compounds previously administered to schizophrenics, only L-methionine (Pollin et al, 1961) and betaine (Brune and Himwich, 1963) were reported to give such a reaction. Although the reaction to betaine has yet to be confirmed, betaine and L-methionine have in common methyl donating properties.

In spite of the finding that L-methionine induced an acute schizophreniform psychosis in a proportion of chronic schizophrenic patients, and despite the fact that a disturbance in L-methionine

metabolism has been reported in schizophrenics (Israelstam et al, 1970), it cannot be assumed that this resulted from faulty trans-methylation, since methionine is concerned in other metabolic reactions. Irrespective of the mechanism by which L-methionine produced this reaction, two possibilities about its pathogenesis exist: (i) that it represents an exacerbation of the schizophrenic process, i.e. that L-methionine "converts" "chronic symptoms" into subacute or acute, or (ii) that it is a non-specific psychosis which is superimposed on the schizophrenic illness. Although such a psychosis was not witnessed when normals were given L-methionine (Alexander et al, 1963; Harper et al, 1970), since schizophrenics may have a disordered metabolism, this could explain the discrepancy.

In the present investigation it was shown that L-methionine could induce what was considered to be an acute schizophreniform psychosis in some chronic schizophrenic patients. This psychosis was clinically distinct from another reaction which preceded it in some reactors, and was considered to be toxic in nature. However, this cannot be regarded as conclusive, since judgments made solely upon phenomenology are obviously subject to the prejudices of the rating psychiatrists. Although the clinicians in this study were in agreement, as reported, it is possible that other clinicians might have come to different conclusions, especially when diagnostic criteria in schizophrenia differ widely among psychiatrists.

The alleged therapeutic effect of L-methionine in schizophrenia previously reported was not observed in the present study. The two reported reactors who were thought to have had a minor long term

LSD not
a methyl
dross

- not another
impression

improvement were recommenced on phenothiazines immediately after the experiment, which could have affected the clinical course of the illness, and minor fluctuations of this nature are to be expected during a schizophrenic illness.

(B) BIOCHEMICAL

As mentioned earlier, the HMPG and VMA values were calculated as $\mu\text{g}/24$ hours. The precautions enforced during the urine collections obviated the need to present the values as $\mu\text{g}/\text{mgm. creatinine}$; this would have been unreliable due to the effect of L-methionine on the nitrogen balance in the body (Phear et al, 1956), which in turn would have affected creatinine clearance. The 24 hour urinary excretion of HMPG was not found to be dependent on urine volume; thus the fluctuations detected most probably gave a rough estimate (since fluctuations in peripheral NA stores would influence the levels of total urinary HMPG) of the rate of central NA metabolism. The same argument holds for VMA 24 hour urinary levels, which served as a rough index for peripheral NA metabolism.

Schildkraut et al (1964) and Kopin et al (1961) found that MAO-inhibitors decreased urinary levels of VMA and HMPG respectively. In addition, the former group found that NM and NA were increased after the administration of MAO-inhibitors, indicating that the MAO-inhibitors block deamination (intracellularly), thus preventing further catabolism of brain amines. Since MAO-inhibitors were not used in this experiment, theoretically an increase in the O-methylation of NA produced by L-methionine loading would have been paralleled by an increase in the urinary levels of VMA and HMPG.

The failure to detect any such changes did not, however, rule out the possibility that L-methionine might have aggravated the postulated aberration of transmethylation by increasing the body methylating pool. This is because (i) both VMA and HMPG are O-methylated metabolites of noradrenaline; thus the "toxin" might have been an N-methylated derivative of tryptamine, histamine, any of the polyamines (little is known about their role in brain metabolism) or a methylated nucleic acid. Schizophrenics, by virtue of a possible transmethylation defect, might have failed to respond (to L-methionine) by an increase of normally occurring methylated metabolites. This is of special interest since no comparative study has been done to establish the effect of L-methionine on VMA and HMPG excretions in non-schizophrenics.

(ii) Despite the precautions taken in standardizing the diet and keeping the environmental factors as constant as possible, inevitable social and psychological stresses could have caused fluctuations in VMA (Weil-Malherbe, 1967) and HMPG excretion; this could have masked statistically significant changes induced by L-methionine feeding. This was especially observed in the case of VMA where 24 hour urinary levels fluctuated, showing an increase after any stress condition (i.e. glycine or methionine). Again, no study has yet been done to examine the effects of stress on HMPG excretion in normals.

(iii) Although L-methionine had been shown in in vitro experiments to donate methyl groups to a number of metabolites in the body (including catechol metabolites), giving it in excess might not increase methylation. This could have been due to a rate limiting factor in the body which checks

the rate of methylation under a non-physiological condition; rate limiting factors are known to exist in many metabolic pathways in the body. However, schizophrenics might have defective demethylation and hence a slower clearance of the body methylating pool.

Since there were no statistically significant changes in VMA and HMPG excretions, there were no correlations between increased methylation (as reflected by increases in VMA and HMPG urinary levels) and the clinical reaction to L-methionine. Even if a correlation was demonstrated, a cause and effect relationship could not have been postulated because, as mentioned earlier, L-methionine has other metabolic effects in the body.

However, the above findings are not contradictory to the "transmethylation hypothesis". Further study geared to a better understanding of L-methionine metabolism in normals and schizophrenics is required. A more exhaustive and direct approach to the problem of methylation and demethylation reactions should, therefore, be pursued.

Because MAO-inhibitors were not given during the experiment, and there was no significant increase in the levels of urinary HMPG on L-methionine loading, it was possible to compare the levels of urinary HMPG in the chronic schizophrenics with those of the acute schizophrenics and normals.

The difference in urinary levels of HMPG between acute schizophrenics and normals could have been due solely to the effects of stress and physical agitation in the acute population since (1) there was a difference between the acute and chronic groups of schizo-

phrenics, and (ii) there was no difference between the chronic schizophrenics and the controls. However, some of the acute schizophrenics were not overtly or unduly overactive physically and stress phenomena could have occurred in all three groups. Moreover, those chronic schizophrenics who reacted to L-methionine showed acute symptoms and were physically overactive, yet did not show any increase in urinary HMPG.

This preliminary finding needs further investigation using a longitudinal study of the excretion of HMPG in a larger sample of schizophrenics and normals.

SECTION IV

ANIMAL STUDIES

L-METHIONINE - A METHYL DONOR FOR O-METHYLATION OF CATECHOLAMINE CATABOLITES, IN VIVO.

M A T E R I A L S A N D M E T H O D S

(A) EXPERIMENTS' DESIGN

The four main objectives of this study were to (i) demonstrate the transfer of the methyl group from L-methionine to catechol-O-methylated catabolites, in brain, (ii) demonstrate such a transfer in total body, (iii) show that the methods used in the separation and identification of these O-methylated catabolites are valid, and (iv) demonstrate the authenticity of the O-methylated catabolites.

EXPERIMENT I

Three groups of male Wistar albino rats, weighing 150 g each, were taken.

Group I (9 rats) - each was injected through the tail vein with 25 μ Ci of L-methionine (methyl-C¹⁴) (60 mCi/mMol)* in 1 ml. of physiologic saline. The rats were killed two hours after the injection by cervical fracture, decapitated, and their brains removed.

*L-methionine (methyl-C¹⁴) - CFA.152, The Radiochemical Centre, Amersham.

Group II (2 rats) - each was anaesthetized with ether, then injected intraventricularly (Noble et al, 1967) with 0.5 μ Ci of DL-noradrenaline (carbinol C¹⁴) (25 mCi/mMol)** in 20 μ l of Merles solution. The rats were killed one hour after the injection by cervical fracture, decapitated, and their brains removed.

Group III (3 rats) - each was injected through the tail vein with 25 μ Ci of L-methionine (methyl-C¹⁴) (60 mCi/mMol) in 1 ml. of physiologic saline. The rats were then placed in metabolic cages and their urine collected for the 24 hour period following the injection. No plasma expanders were used and the rats were given water ad lib.

EXPERIMENT II - Consisted of Groups I and III only.

(B) PRELIMINARY ASSAYS

(1) Brain Assay: Each brain (from Group I and II) was homogenized in an all-glass homogenizer with chilled 0.4N PCA (0.05% ascorbic acid and 0.1% EDTA) using 4 ml/g brain. The homogenate was spun in a refrigerated centrifuge (4°C) for 15 minutes at 20,000 g. At this stage an 0.2 ml. aliquote of the supernatant was dissolved in 4 ml. ethanol:methanol (3:1) and 10 ml. of Toluene (0.426% PPO, 0.011% POPOP) scintillator and counted by liquid scintillation to assess the success of the intravenous (Group I) or intraventricular (Group II) injection. The supernatants from Group I and II were

** DL-noradrenaline (carbinol C¹⁴), DL-bitartrate -
The Radiochemical Centre, Amersham.

pooled separately; the two mixtures were adjusted to pH4 (glass electrode) with 5N and 1NKOH and cooled to 4°C for 10 minutes to precipitate the potassium perchlorate. They were then centrifuged for 3 minutes at 4,000 g and the supernatants decanted and adjusted to pH1 (glass electrode) with concentrated HCl.

(2) Urine Assay: The 24 hour urines were pooled and adjusted to pH1 (glass electrode) with concentrated HCl.

(C) SEPARATIVE PROCEDURES

(1) Phenolic Acids: Each of the two supernatant mixtures (pH1) (from Groups I and II) and the combined urine sample (pH1) (from Group III) was extracted with double volume of ethyl acetate by shaking for 5 minutes and centrifuging at 2,000 g for 5 minutes; after removing the ethyl acetate the extraction was repeated twice with two further double volumes of ethyl acetate. The aqueous phase was saved for the estimation of the alcohols and O-methylated amines. The combined organic phase (ethyl acetate) was extracted once with one-sixth its volume of $\text{1MK}_2\text{CO}_3$ buffer (pH10) by shaking the mixture for 5 minutes and centrifuging for 5 minutes at 2,000 g. The buffer layer (containing the acids) was adjusted to pH1 (glass electrode) with concentrated HCl and extracted with double volume of ethyl acetate; the extraction was done three times. At that stage (i) 10 µg of authentic unlabelled VMA and HVA were added to the combined ethyl acetate fractions from Groups I and III, and 10 µg of VMA to that from Group II. (ii) The combined ethyl acetate fraction from Group I was divided into three equal portions.

Each combined ethyl acetate fraction from Groups II and III and the three portions from Group I were blown down under a stream of nitrogen to \approx 0.2 ml. and stored at -20°C until applied to chromatography paper.

Recovery from Extraction: Two samples (in duplicate) of 5 ml. water were taken; to one was added 5 μg VMA and the other 5 μg of HVA. The samples were adjusted to pH1 with conc. HCl and extracted in the same way as in the method (first ethyl acetate - K_2CO_3 buffer - second ethyl acetate). The combined second ethyl acetate fraction from each sample was blown down to damp dryness with nitrogen. Each residue was dissolved in 3 ml. distilled water, 2 ml. of which was taken for the fluorometric determination of HVA or VMA by the FeCl_3 oxidation reaction.

The overall recovery of VMA and HVA, under these conditions, was $80 \pm 5\%$ (4) (mean \pm SD (no. of observations)).

(2) Alcohols from O-methylated amines: The aqueous phase of each sample was adjusted to pH5 (glass electrode) with 5N and LNKO₂, and reduced to 1 ml. under vacuum at 37°C .

(i) Hydrolysis: To each concentrate were added (i) 9 mg. of Helicase enzyme (powder form), (ii) 0.1 ml. of 1M sodium acetate buffer (pH5), (iii) 10 μg of authentic unlabelled HMPG, HMPT, NM, M and MTYR* for Groups I and III, and HMPG, NM and M for Group II, and (iv) two drops of chloroform. The mixture was incubated at 37°C for 24 hours then centrifuged for 5 minutes at 3,500 g and the supernatant decanted.

* MTYR - 3-methoxy tyramine (3-methoxy dopamine).

(ii) Column Chromatography

(a) Alcohols: Each supernatant was adjusted to pH7.5 (glass electrode) with 1N and 0.1N KOH and passed over a column (4 x 0.7 cm.) of Bio-Rad AGI - X4 untreated resin (chloride form, 200-400 mesh). The column was washed with 2 ml. of distilled water which, together with the effluent, was saved for the determination of the O-methylated amines. The alcohols (only HMPG in Group II) were eluted off the column with 8 ml. of distilled water; the eluate from Group I was divided into three equal portions.

Each eluate from Group II and III and the three portions from Group I were reduced to \surd 0.2 ml. under vacuum*at 37°C and stored at -20°C until applied to chromatography paper.

Column Recovery: Two samples (in duplicate) of 5 µg HMPG in 1 ml. of distilled water were passed over columns (4 x 0.7 cm.) of AGI-X4 resin. The columns were washed and eluted with 2 and 8 ml. of distilled water respectively. Each eluate was reduced to damp dryness under vacuum and the residue dissolved in 3 ml. distilled water, 2 ml. of which was taken for the fluorometric determination of HMPG by the FeCl₃ oxidation method. The overall recovery was 78 ± 4% (4) (mean ± SD (no. of observations)).

(b) O-Methylated Amines: The pH of each mixture (3 ml.) of effluent and wash from the Bio-Rad resin was checked and readjusted to pH 7.5 (glass electrode) if necessary. It was passed over a column (10 x 0.7 cm.) of Amberlite CG 50 resin (NH₄⁺ form, pH7.5). The column was washed with 10 ml. of distilled water and the O-methylated amines were eluted off the column with 15 ml. of 3N NH₄OH.

* Ascorbic acid⁺ (\surd 200 µg) was added to each sample before it was reduced in volume.

⁺ L-Ascorbic acid - Analar Reagent (BDH).

At this stage the eluate from Group I was divided into three equal portions, and together with the eluates of Groups II and III, was reduced to ≈ 0.2 ml. under vacuum** at 37°C ; they were stored at -20°C until chromatography.

CG 50 Column Recovery: The column adsorbs NA, dopamine and the O-methylated amines. Using labelled NM and fluorometric measurement of MTYR and M, according to the method of Axelrod et al (1958), it was found that the column size in relation to the sample, wash and eluate volumes was important for obtaining maximal recovery. Eluting the O-methylated amines with $3\text{N NH}_4\text{OH}$ gave uniform recoveries from the column. A sample volume of 1 or 3 ml. required a column size of 4×0.7 cm. OD or 10×0.7 cm. OD respectively. Under these conditions, washing the column with water only eluted a small proportion of the O-methylated amines, thus:

The effluent contained $\# 5 \pm 2\%$ of the O-methylated amines.

The 10 ml. water wash contained $\# 9 \pm 3\%$ of the O-methylated amines.

The 15 ml. $3\text{N NH}_4\text{OH}$ eluate contained $\# 86 \pm 5\%$ of the O-methylated amines.

+ The value represents the mean of:

1 sample (duplicate)	- containing H^3NM ($\approx 20,000$ DPM)	} in 3 ml. water (pH 7.5)
1 sample (duplicate)	- containing M ($20 \mu\text{g}$)	
1 sample (duplicate)	- containing MTYR ($20 \mu\text{g}$)	

* (mean \pm SD (6)).

When the eluate volume was less than 15 ml. there was a decrease in recovery.

** Ascorbic acid⁺ ($\approx 200 \mu\text{g}$) was added to each sample before it was reduced in volume.

Since $90 \pm 5\%$ of the O-methylated amines are recovered in the effluent ($\approx 60\%$) and wash ($\approx 25\%$) of the AG1-X4 column (see HMPG method - specificity of isolation procedure), the overall recovery of these amines was $83 \pm 5\%$.

L-methionine and S-adenosylmethionine - recoveries through the separative procedures:

The following samples were tested for recoveries through the method:-

1. Ethyl acetate extraction: Two samples, one containing labelled L-methionine (50,000 DPM) and the other labelled S-adenosylmethionine (50,000 DPM) in 2 ml. of water were adjusted to pH1. They were extracted three times with double volume ethyl acetate. One ml. of each aqueous phase and 6 ml. of the organic phase were dissolved in two separate 10 ml. fractions of Toluene Triton X (PPO, POPOP) scintillator and counted by liquid scintillation against appropriate blanks.
2. Bio-Rad AG1-X4 column: Two samples, one containing labelled L-methionine (50,000 DPM) and the other labelled S-adenosylmethionine (50,000 DPM) in 1 ml. of water were adjusted to pH7.5. They were passed over columns (4 x 0.7 cm. OD) of AG1-X4 resin. Each column was washed with 2 ml. of water and eluted with 8 ml. of water. One ml. of each effluent, wash and eluate was dissolved in a separate 10 ml. fraction of Toluene Triton X (PPO, POPOP) scintillator and counted by liquid scintillation against water blanks.

3. Amberlite CG 50 column: Two samples, one containing labelled L-methionine (50,000 DPM) and the other labelled S-adenosylmethionine (50,000 DPM) in 3 ml. of water were adjusted to pH7.5. They were passed over columns (10 x 0.7 cm. OD) of CG 50 resin. Each column was washed with 10 ml. of water and eluted with 15 ml. of 3N NH₄OH. One ml. of each effluent, wash and eluate was dissolved in a separate 10 ml. fraction of Toluene Triton X (PPO, POPOP) scintillator and counted by liquid scintillation against appropriate blanks.

Results

1. L-methionine and S-adenosylmethionine - organic phase - 1%
aqueous phase - 99%

2. L-methionine - effluent 39%
wash 6%
eluate 1%

S-adenosylmethionine - effluent 54%
wash 32%
eluate 5%

* remainder adsorbs to resin.

		<u>S-adenosyl-</u> <u>Methionine</u>	<u>L-methionine</u>
3.	L-methionine and S-adenosylmethionine	- effluent 4%	65%
		wash 5%	34%
		eluate 1%	0.35%

(D) IDENTIFICATION

(1) Phenolic Acids

(i) Paper chromatography: Each 0.2 ml. ethyl acetate concentrate (Groups II and III; three portions of Group I) was applied, under a stream of nitrogen, to a separate sheet of Whatman No. 1 chromatography paper (46 x 50 cm.) (Appendix III (b)). Then the sides of each tube were washed with 0.1 ml. of methanol which was also applied to the paper on the same spot as the sample. The origin (point of application) was at one corner of the paper, 10 cm. from both edges (Appendix III (b)). Markers of authentic VMA and HVA in Groups I and III, and VMA in Group II were applied on the same spot as each sample.

Using the "Two dimensional descending technique": The paper chromatograms were placed in sealed chromatography tanks* containing the first solvent system and left to equilibrate with the solvents' vapours for at least six hours. They were then developed, removed from the tanks and dried with nitrogen for six to eight hours. The papers were rotated 90°, placed in other sealed tanks containing the second solvent system, and allowed to equilibrate for six hours with the solvents' vapours. They were then developed, removed from the tanks and dried with nitrogen.

To visualise the mixed sample and marker spots the developed chromatograms were sprayed with paranitroaniline: 10% sodium nitrite (10:1) and allowed to dry (Figures 11-a,b,c; 12-iii; 13-iii).

* All the chromatography tanks used were saturated with nitrogen before sealing.

The combinations of solvent systems used were as follows:

- | | | |
|--|--|------------------------------------|
| 1. 1st System - Isobutyl methyl ketone:4% formic acid
(10:1 v/v) - 2 phase (organic phase used)
<u>Developing time: 5 hours.</u>
(Appendix V,1,a) | 2nd System - Chloroform:methanol:ammonia (0.88)
(12:7:1 v/v) - single phase
<u>Developing time: 5 hours.</u> | } <u>Groups</u>
I,II
and III |
| 2. 1st System - Isopropyl alcohol:ammonia (0.88):
water (20:1:4 v/v) - single phase
<u>Developing time: 20 hours.</u>
(Appendix V,1,b) | | |
| 2nd System - Butanol:ethanol:water
(4:1:1 v/v) - single phase
<u>Developing time: 17 hours.</u> | | } <u>Group</u>
I |
| 3. 1st System - Butanol:pyridine:water
(14:4:5 v/v) - single phase
<u>Developing time: 16½ hours.</u>
(Appendix V,1,c) | | |
| 2nd System - Butanol:acetic acid (glacial): water:
dichlorethane (1:1:1:3) v/v) - 2 phase
(organic layer used)
<u>Developing time: 5 hours.</u> | | |

Two phase solvent system: The mixture was shaken and left to settle, forming two layers. Most of the organic layer was poured into the chromatography tank and the remainder was used to develop the chromatogram. The aqueous layer was poured into a trough, placed in the upper part of the tank and allowed to flow down on a sheet of chromatography paper, the upper edge of which was dipped in the trough. The lower edge of the paper hung into another trough placed at the bottom of the tank to collect the down flowing aqueous phase, thus preventing it from mixing with the organic phase.

(ii) Liquid Scintillation Counting: An area measuring 10 cm. in the direction of the second solvent flow and 5 cm. wide was drawn over each spot and then cut into ten strips of 1 x 5 cm. Each strip was immersed in 4 ml. of Toluene (PPO and POPOP) scintillator and counted by liquid scintillation against 1 x 5 cm. blank strips cut from strips of paper which were developed in the same solvent systems used for the sample chromatograms, then sprayed with a mixture of paranitroaniline and sodium nitrite (Figures 11-a,b,c; 12-iii; 13-iii).

(2) Alcohols

(i) Paper Chromatography: Each 0.2 ml. concentrated eluate from the Bio-Rad resin (Groups II and III; three portions of Group I) was applied to a separate 46 x 50 cm. sheet of Whatman No. 1 chromatography paper in the same way as in the case of the acids, except for the markers, where authentic HMPG and HMPT in Groups I and III and HMPG in Group II were applied instead.

The same chromatography technique and solvent systems used for the acids were utilized, i.e. -

Combination (1) for Groups I,II and III (Appendix V,1,a).
Combinations (2) and (3) for Group I (Appendix V,1,b,c).

The developed chromatograms were sprayed with a mixture of paranitroaniline and sodium nitrite and allowed to dry (Figures 11 (I)-a,b,c; 12-ii; 13-ii).

(ii) Liquid Scintillation Counting: The method for cutting out the spots and counting their radioactivity was the same as that used for the acids (Figures 11 (I)-a,b,c; 12-ii; 13-ii).

(3) O-Methylated Amines

(i) Thin Layer Chromatography: Three of the 0.2 ml. concentrated eluates from the Amberlite resin (Groups II and III; one of the three portions from Group I) were applied under a stream of nitrogen on three separate silica gel thin layer plates (20 x 20 cm.)*. The sides of each tube were washed with 0.1 ml. methanol which was applied on the same spot as the sample itself. The point of application was at the corner of the plate, 3 cm. from each edge (Appendix V,2,a). Markers of authentic M, NM and MTYR in Groups I and III, and M and NM in Group II were applied on the same spot as each sample.

Using the two dimensional ascending technique: the three plates were placed in a sealed chromatography tank containing the first solvent system of Isopropyl alcohol:ammonia 5% (4:1 ^{v/v}) (single phase) and allowed to equilibrate with the solvents' vapours for three hours. They were then developed until the solvent front reached the upper edge of the plates. The plates were removed from the tank and dried with nitrogen for six to eight hours. They were rotated 90° and placed in another sealed tank containing the second solvent system of Benzene:acetic acid (glacial):water (62.5:36:1.5 ^{v/v}) (single phase). After an equilibration period of three hours with the solvents' vapours, the plates were developed until the solvent front reached the upper edge of the plates. They were removed from the tank, dried and redeveloped twice more in the second solvent system, allowing each time for the solvent front to reach the upper edge of the plates (Appendix V,2,a).

* TL - Silica gel precoated plates - 20 x 20 cm., 0.25 mm. layer thickness (without fluorescence indicator) - E. Merck, A.G. Darmstadt.

After the third development the plates were dried, the mixed marker and sample spots were visualised by spraying the plates with a mixture of paranitroaniline and sodium nitrite (Figures 11 (II)-a; 12-i; 13-i).

(ii) Paper Chromatography: The remaining two concentrated portions (from the eluate of the Amberlite resin) of Group I were applied, under a stream of nitrogen, to two separate sheets of Whatman No. 1 chromatography paper (46 x 50 cm.) in the same way as in the case of the acids and alcohols except for the markers, where NM, M and MTYR were used instead.

Using the two dimensional descending technique, the following two combinations of solvent systems were used:-

1. 1st System - Butanol:acetic acid (glacial):water
(4:1:1 ^v/v) - single phase
Developing time: 18 hours.

(Appendix V,2,b)

2nd System - 20% KCL
Developing time: 4 hours.

2. 1st System - Butanol:ethanol:ammonia 0.88
(4:1:1 ^v/v) - single phase
Developing time: 17 hours.

(Appendix V,2,c)

2nd System - Nitroethane 70%:acetic acid (glacial)
(9:4 ^v/v) - single phase
Developing time: 5 hours.

Group I

The developed chromatograms were sprayed with a mixture of paranitroaniline and sodium nitrite; the mixed sample and marker spots were visualised (Figure 11 (II)-b,c).

(iii) Liquid Scintillation Counting

Thin Layer Chromatography: An area measuring 7 cm. in the direction of the second solvent flow and 4 cm. wide was drawn over each spot, then scraped in seven portions of 1 x 4 cm. Each portion was suspended in a mixture of Thixotropic gel and 10 ml. Toluene Triton X (PPO, POPOP) scintillator and counted by liquid scintillation against blanks. The blanks were 1 x 4 cm. scrapings from a plate developed and sprayed in the same way as the sample plates were (Figures 11 (II)-a; 12-i; 13-i).

Paper Chromatography: The developed chromatograms were cut and counted in the same way as in the case of the acids and alcohols (Figure 11 (II)-b,c).

RESULTS

1. All values of radioactivity were corrected for quenching by the channel ratio method, thus expressed as DPM.
2. Using the "internal standard method", it was found that the amber-yellow colour of the spots, obtained from spraying the developed chromatogram with paranitroaniline:sodium nitrite, did not cause any quenching of the "fluor".
3. After correcting for percentage recoveries from the method, the values were expressed as per cent of the intra-group total radioactivity for (i) NA and (ii) Dopamine O-methylated catabolites,

e.g.
$$\frac{\text{VMA activity (DPM)}}{\text{VMA + HMPG + NM + M activity (DPM)}} \times 100$$

TABLE 9

CATABOLITES OF NA AND DOPAMINE

AS PER CENT OF TOTAL O-METHYLATED CATABOLITES

		NA Catabolites				Dopamine Catabolites			
		VMA	Hydrolysed HMPG	NM	M	HVA	HMPY	MTYR	
Group I	%*	Insig-nificant	45	48	7	73	Insig-nificant	27	Figure 11, 11 (I,II)
Group II	%	10	59	29	2	-	-	-	Figure 12-i,ii iii
Group III	%**	86	5	6	3	76	Insig-nificant	24	Figure 13-i,ii iii

* Group I - values represent mean per cent obtained from the
 (i) three chromatography combinations.
 (ii) two experiments.

** Group III - values represent mean per cent obtained from the two experiments.

Group I - Endogenous catechol-O-methylated catabolites in brain after intravenous injection of C¹⁴-L-methionine.

Group II - Catabolism of C¹⁴-NA in brain after intraventricular injection.

Group III - Endogenous catechol-O-methylated catabolites in 24 hour urine after intravenous injection of C¹⁴-L-methionine.

FIGURE 11

Group I C¹⁴-METHYL-L-METHIONINE -INTRAVENOUS-BRAIN ASSAY
ACIDS

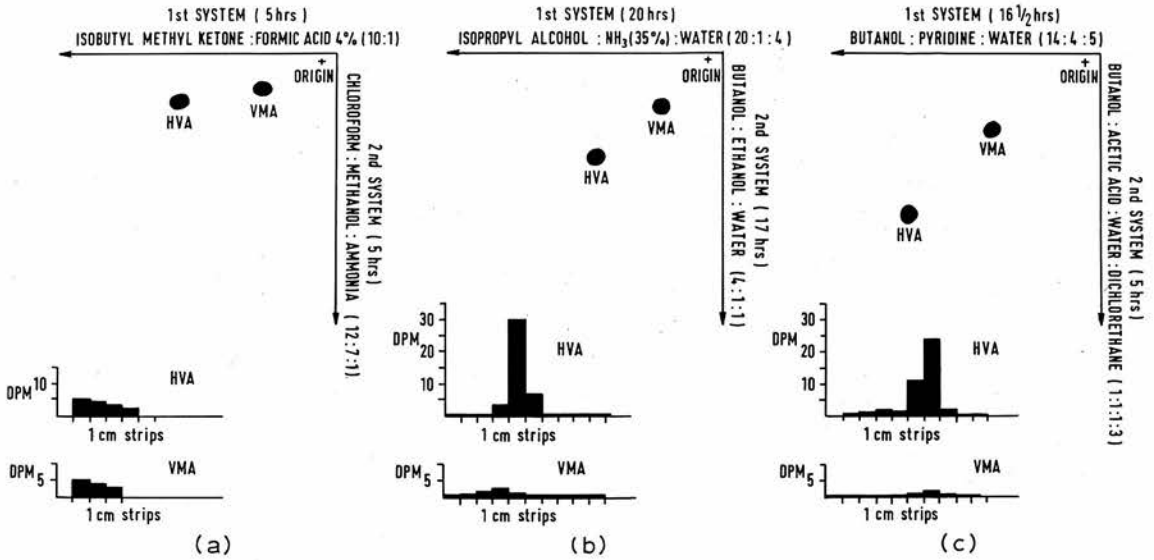


Figure represents the acid catabolites HVA and VMA of endogenous dopamine and NA respectively in brain. It shows that the spots corresponding to HVA in the three chromatographic solvents (a,b,c) have more radioactivity than those of VMA which have background levels of activity.

The spots were visualized by spraying with diazotised paranitroaniline.

Whatman No. 1 chromatography paper was used.

The results represent an average of two experiments.

FIGURE 11 (I)

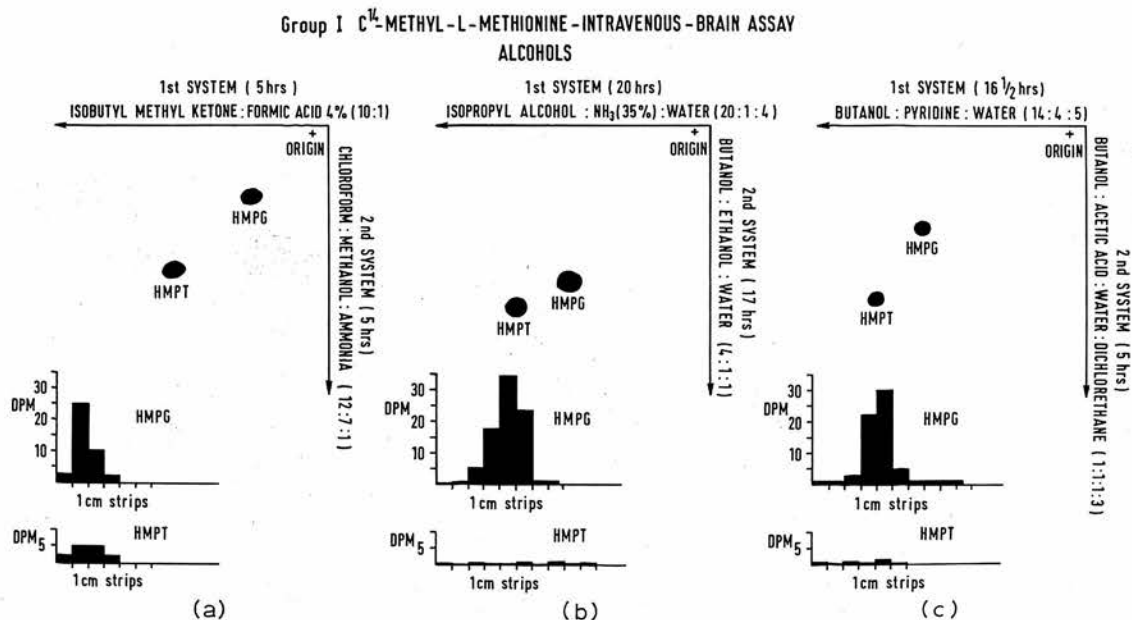


Figure represents alcohol catabolites - HMPT and HMPG - of endogenous dopamine and NA respectively in brain. It shows that the spots corresponding to HMPG in the three chromatographic solvents (a,b,c) have appreciable levels of radioactivity while those of HMPT have background levels of activity.

The spots were visualized by spraying with diazotised paranitroaniline.

Whatman No. 1 chromatography paper was used.

The results represent an average of two experiments.

FIGURE 11 (II)

Group I C^{14} -METHYL-L-METHIONINE - INTRAVENOUS - BRAIN ASSAY
O-METHYLATED AMINES

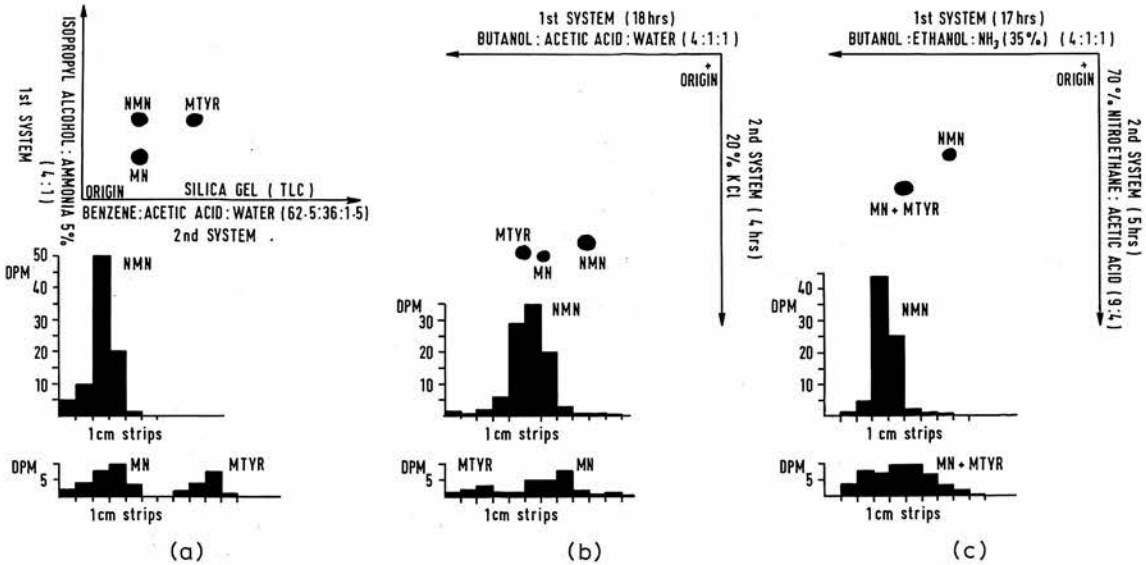


Figure represents the O-methylated amine catabolites - NM and M; MTYR of endogenous NA and dopamine respectively in brain. It shows that the high levels of radioactivity in the three chromatographic solvents (a,b,c) correspond to the NMN spots. The spots corresponding to MN and MTYR have much lower activity.

The spots were visualized by spraying with diazotised paranitroaniline.

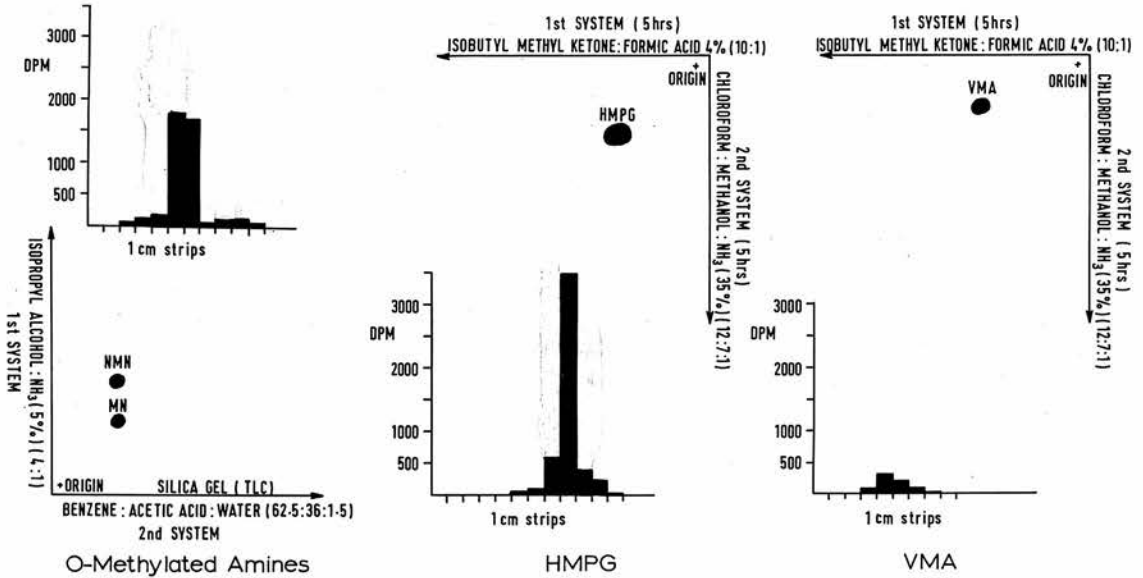
(a) Thin layer precoated silica gel plates (20 x 20 cm.) were used.

(b,c) Whatman No. 1 chromatography paper was used.

The results represent an average of two experiments.

FIGURE 12

Group II C^{14} -DL-NORADRENALINE - INTRAVENTRICULAR - BRAIN ASSAY



(i)

(ii)

(iii)

Figure represents the O-methylated catabolites of exogenous NA in brain. It shows that the highest radioactivity corresponds to the HMPG (ii) then NMN (i) spots. The spot corresponding to VMA has much lower activity, while that of MN has the least.

The spots were visualized by spraying with diazotised paranitroaniline.

(i) Thin layer precoated silica gel plate (20 x 20 cm.) was used.

(ii, iii) Whatman No. 1 chromatography paper was used.

FIGURE 13

Group III C^{14} -METHYL-L-METHIONINE - INTRAVENOUS -
24 hr URINE

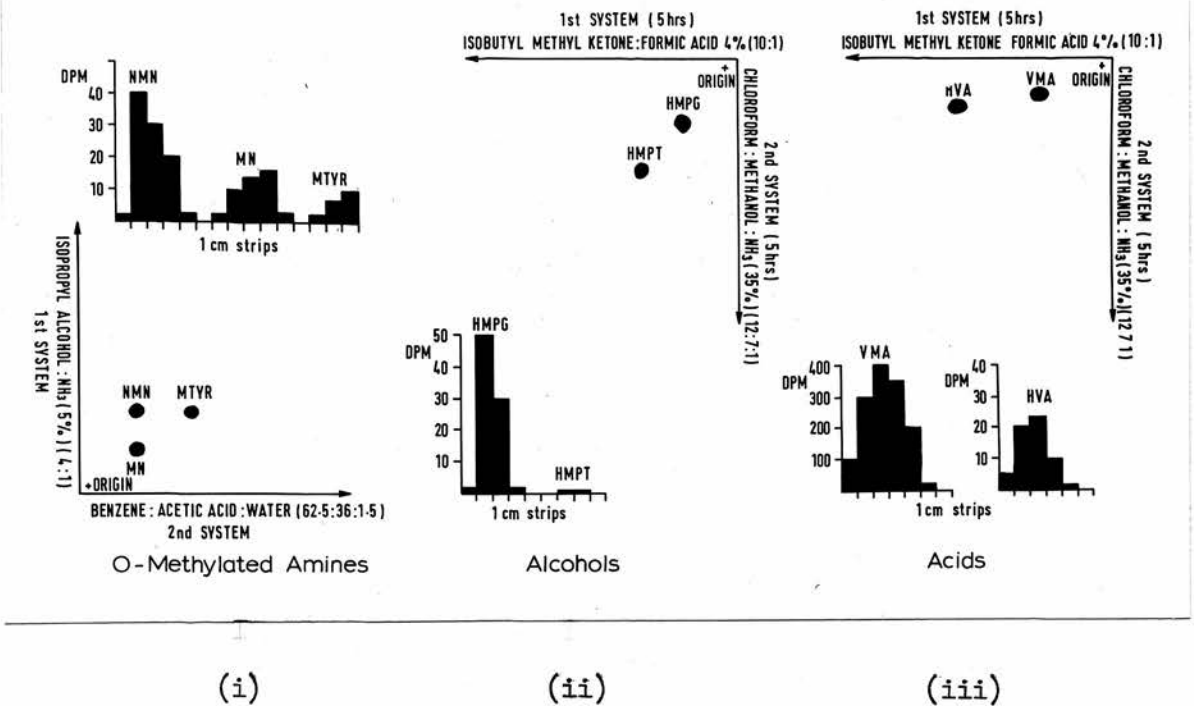


Figure represents the O-methylated catabolites of endogenous NA and dopamine in urine. It shows that the area corresponding to VMA has the highest radioactivity while HMPT is found in negligible amounts.

The results represent an average of two experiments.

The spots were visualized by spraying with diazotised paranitroaniline.

(i) Thin layer precoated silica gel plate was used.

(ii, iii) Whatman No. 1 chromatography paper was used.

DISCUSSION

The results of this study show that the separative and identification procedures were adequate for isolation of the various catechol-O-methylated catabolites. This was clearly seen in the control group (Group II), where C^{14} -NA was injected intraventricularly. In this group percentages of the different O-methylated NA catabolites tended to fall within the range reported by other workers (Diagram III), allowing for the fact that all the present results express the percentages of the O-methylated catabolites only. In other words, the percentages reported in the experiment were calculated from the sum total radioactivity of the O-methylated catabolites only; thus they would be higher than percentages of total catabolites (O-methylated and dihydroxy).

Rutledge and Jonason (1967) used rabbit brain slices to study the catabolism of exogenous and endogenous NA. The former was assessed by incubating the brain slices with H^3 -NA and the latter by incubating with C^{14} -dopamine and isolating the catabolites of the newly formed C^{14} -NA. They reported that 53 per cent of exogenous NA total catabolites were HMPG and DHPG, with predominance of the latter, while NM accounted for 20 per cent and VMA 7 per cent. On the other hand, the majority of the newly formed C^{14} -NA was catabolized into HMPG and DHPG, with the latter occurring in quantities twice that of the former; NM and VMA were found in negligible amounts. The authors suggested that these observations support the view that endogenous NA is initially catabolized

by intraneuronal MAO, whereas exogenous NA can be catabolized initially by either MAO or extraneuronal COMT. The high values of DHPG obtained were probably due to the fact that (i) Rutledge and Jonason determined free HMPG and DHPG only. Schanberg et al (1968) reported that rat brain slices are capable of forming HMPG conjugate. Thus, if there is no species difference a similar situation might occur in rabbit brain slices. (ii) Although there is a high concentration of S-adenosylmethionine (active methyl groups) in brain, there exists a fast and continuous turnover of S-adenosylmethionine in the body, with constant replenishment of tissue stores (Baldessarini, 1967). Therefore, it is quite possible that in vitro S-adenosylmethionine in brain slices is not at an optimal concentration for enhancing maximum O-methylation by COMT. Schanberg et al (1968) also studied the catabolism of exogenous NA in vivo and reported that in rat brain, 90 minutes after intracisternal injection of H^3 -NA, 44 per cent was catabolized and of this 36 per cent appeared as HMPG conjugate, while NM accounted for 4 per cent and VMA and free HMPG 3 per cent. Moreover, there was a decrease in NM with time after the injection; hence NM accounted for 7 per cent at 33 minutes.

On the other hand, R. Sugden (1970 - personal communication) found that 68 per cent of intraventricular C^{14} -NA was catabolized by brain one hour after injection. He reported that 26 per cent of the catabolized NA was identified as HMPG conjugate while NM and VMA accounted for 14 per cent and 4 per cent respectively.

Considering the difference in the time interval of labelled

NA injection and assay between the Schanberg et al experiment and the present one, the results of both experiments (Sugden; Schanberg et al), when expressed as per cent of total catabolites, are also broadly similar to the present values in Group II, except for NM (Diagram III). The latter is higher than the values reported above and by Glowinski et al (1965, 1966) who also studied, in vivo, the catabolism of exogenous NA in brain.

The percentages of NA and dopamine catabolites in brain and urine (Groups I and III) after intravenous C^{14} -L-methionine represent the catabolism of endogenous catechols. To date, there is no detailed study of in vivo catabolism of endogenous NA in brain. Schanberg et al (1968a) and Sharman (1969) were able to detect (in vivo) in rat and mouse brains endogenous HMPG, the majority of which was in the conjugate form. On the other hand, endogenous VMA in brain has not been detected in vivo (Andén et al, 1964; Laverty and Sharman, 1965) and only in negligible amounts in vitro (Rutledge and Jonason, 1967 - vide supra). In urine endogenous VMA and HMPG have been detected (Pisano et al, 1962; Wilk et al, 1967). However, VMA was found to be the major catabolite of exogenous NA and excreted at a more rapid rate than HMPG (Maas and Landis, 1966, 1968). Provided the catabolism of endogenous NA in brain and body is similar to that of exogenous NA, the major catabolites in brain and periphery are HMPG and VMA respectively (Diagram III, Groups I and III).

The high levels of endogenous NM in brain, reported in this study (Diagram III, Group I), perhaps reflects the effect of time

between injection and assay which is less important when studying NA catabolism in vitro. Both in vitro and in vivo NM is further catabolized to the various deaminated derivatives. However, in vivo, the percentage of NA catabolites found at any one time is the result of possibly unequal efflux from brain into the general circulation (Glowinski and Baldessarini, 1966; Werdinius, 1967). NM does not readily cross the blood brain barrier (Maas and Landis, 1966; Schanberg et al, 1967) and it is possible that the deaminated catabolites are eliminated from brain at a more rapid rate than NM; thus there may be an initial (i.e. 1-2 hours) accumulation of NM in brain which is cleared as NM gets further deaminated by MAO. This might also explain why Rutledge and Jonason (1967) could not detect endogenous NM from newly formed C¹⁴-NA in 'in vitro' brain preparations while Häggendal (1962) was able to detect it in brain, in vivo.

The low, but significant, levels of M in brain (Diagram III, Group I) are broadly similar to those reported by Ciaranello et al, (1969). They found that brain tissue can convert NA to adrenaline which, in turn, is catabolized to M; the latter constitutes 4-17 per cent of the combined NM and M fraction, depending upon the species (Barchas et al, 1969). The majority of the M and adrenaline found in brain comes from central stores, since both do not readily cross the blood brain barrier (Barchas et al, 1968; Axelrod et al, 1959c). Probably a greater proportion of endogenous NA is converted to adrenaline than exogenous NA which is rapidly catabolized in brain after injection (Schanberg et al, 1968). This

might explain the much lower percentage of M in Group II, where C^{14} -NA was injected.

The catabolism of dopamine has been studied extensively in the past. Andén et al (1963) found that the major catabolite of endogenous dopamine in brain was HVA; this was later confirmed by Rutledge and Jonason (1967) who found that in rabbit brain slices HVA was 81 per cent of the total catabolites of exogenous C^{14} -dopamine, MTYR accounted for 8 per cent and HMPT was only detected in negligible amounts (Diagram III). Moreover, the only endogenous dopamine catabolites (other than HVA) isolated from brain so far are dihydroxyphenylacetic acid (DOPAC) (Andén et al, 1963a) and MTYR (Carlsson and Waldeck, 1964). These results are at variance with NA where the glycols are the major catabolites. This probably suggests that either the dopamine alcohols are eliminated from brain at a higher rate than the acids and MTYR or the NA deaminated aldehyde is primarily reduced to phenolic glycols while the dopamine aldehyde is oxidized to the phenolic acids. Although little is known about the enzyme system(s) responsible for further catabolism of these aldehydes, Eccleston et al (1966) found that in 'in vitro' brain preparations, serotonin (5HT) is catabolized to 5-hydroxy-acetaldehyde which in turn is mostly oxidized to 5-hydroxyindole-acetic acid (5HIAA) rather than reduced to the alcohol, 5-hydroxy-tryptophol. However, it was found that the ratio of acid/alcohol depends on an intricate balance of co-enzymes (reduced nicotinamide adenine dinucleotide phosphate for reduction and both nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate

for oxidation) which alters the rate of reduction or oxidation. Furthermore, in brain, the reduction depends on the ratio of NADPH_2 (reduced): NADP_1 .

It is interesting to note that, in urine, the per cents of HVA and MTYR are similar to those in brain. However, comparative values by other workers were unobtainable.

Indeed, the experiment was not set out to investigate the rate of catechol amine catabolism, yet the fact that the pattern of predominance of these "presumed" catabolites (Groups I and III) compares with that reported by other workers is a suggestive evidence for their authenticity. This is not to deny that (i) it is very difficult to make any conclusive interpretations when the levels of radioactivity are very low and, therefore, the possible experimental error is high, and (ii) L-methionine donates methyl groups to a host of metabolites in the body, and the radioactivity of these catabolites comes from L-methionine C^{14} -methyl groups. Although using different chromatographic solvents, as adopted in this study, reduces the chances of contamination from other methylated metabolites, it is virtually impossible to rule out all of them. Ideally, a more direct proof would have been to block O-methylation by inhibiting COMT (e.g. pyrogallol) and determining whether the peaks of radioactivity, presumed to be methylated catechols, disappear. However, this approach would have been technically very difficult since pyrogallol, as well as the tropolones, blocks COMT by competitive substrate inhibition, thus competing for the methyl group (Archer et al, 1960; Belleau and

Burba, 1963). This would have resulted in C^{14} labelled methylated derivatives which had to be identified to make sure they did not behave like O-methylated catechol metabolites through the separative procedures, thus acting as contaminants.

The experiment shows that in vivo some of the C^{14} radioactivity, originating from the C^{14} -methyl groups of L-methionine, is isolated from brain and 24 hour urine, and demonstrated, using chromatographic procedures, as probably O-methylated catabolites of NA and dopamine. The percentage of each catabolite falls within the range reported by other workers, thus suggesting evidence for their authenticity.

DIAGRAM III

SUMMARY OF EXOGENOUS AND ENDOGENOUS NA AND DOPAMINE CATABOLISM IN BRAIN AND PERIPHERY
(Results are obtained from the present study and experiments by other workers)

	Free HMPG and DHPG	VMA	NM	M	HVA	HMPG	MTYR
Rutledge and Jonason (1967) Values - % of total catabolites (brain slices)	H ³ -NA	7	20		81	C ¹⁴ -Dopamine	8
	C ¹⁴ -NA (Newly formed)	Negligible	Negligible				
Schanberg et al (1968) Values - % of total catabolites - 90 min. after NA injection (intracisternal)	Hydrolysed HMPG	VMA + HMPG (Free)	9				
	82	7	33 min. 15				
Sugden, R. (1970) Values - % of total catabolites - 1 hour after NA injection (intraventricular)	Hydrolysed HMPG						
	38	6	20				
Present Study Group I - Intravenous C ¹⁴ -L-methionine - Brain assay 2 hours after	Hydrolysed HMPG	Negligible	48	7	73	Endogenous Dopamine	27
	45						
Group II - Brain assay 1 hour after intraventricular injection	59	10	29	2			
Group III - Intravenous C ¹⁴ -L-methionine - 24 hour urine	5	86	6	3	76	Endogenous Dopamine	24

EFFECT OF L-METHIONINE LOADING
ON NORADRENALINE CATABOLISM IN RAT BRAIN

M A T E R I A L S A N D M E T H O D S

(A) EXPERIMENT'S DESIGN

Two groups of albino Wistar rats, each weighing 120 g were put in two separate cages and starved for twelve hours; at the end of that interval -

Group I - Control (5 Rats): Each rat was fed with about 14 g/day of diet (Appendix VI) in pellet form for a period of four days. On the fifth day they were injected intraperitoneally with a suspension of glycine in saline, using 1.5 g of glycine/Kg body weight in 1 ml. saline per rat.

Group II - L-methionine (6 Rats): Each rat was fed with about 14 g/day of a diet containing 6% L-methionine*. The diet was given for a period of four days and on the fifth day each rat was injected intraperitoneally with 1 ml. suspension of 1.5 g L-methionine/Kg body weight in saline.

Three hours after the intraperitoneal injection, each rat from both groups was anaesthetized with ether and injected intraventricularly (Noble et al, 1967) with 0.5 μ Ci of DL-noradrenaline (Carbinol-C¹⁴) (25 mCi/mMol)** in 20 μ l of Merles solution.

* Pellets (identical to those used for Group I) were ground and L-methionine, in the crystalline form, was added. The mixture was then hydrated and reconstituted into pellets similar to the original ones; they were left to dry overnight.

**DL-noradrenaline (Carbinol-C¹⁴) DL-Bitartrate, Radiochemical Center, Amersham.

One hour after the intraventricular injection, the rats were killed by cervical fracture, decapitated and their brains removed.

(B) SEPARATIVE PROCEDURES

Each brain was homogenized, in an all glass homogenizer, with 6 ml. of chilled 0.4N PCA (0.05% ascorbic acid and 0.1% EDTA); the homogenate was spun in a refrigerated centrifuge (4°C) for fifteen minutes at 20,000 g. At this stage, an 0.2 ml. aliquote of the supernatant was dissolved in 4 ml. ethanol:methanol (3:1) and 10 ml. of Toluene (PPO, POPOP) scintillator and counted by liquid scintillation: thus the total radioactivity in the supernatant of each sample was assessed. The remaining supernatant was adjusted to pH4 (glass electrode) with 5N and 1N KOH and cooled at 4°C for ten minutes to remove the excess PCA by precipitating it as the potassium salt. The sample was centrifuged for three minutes at 4,000 g and the supernatant decanted and adjusted to pH1 (glass electrode) with concentrated HCl.

(1) Phenolic Acids: Each supernatant (pH1) was extracted thrice, each time with double volume of ethyl acetate. The aqueous phase was saved for the determination of the glycol conjugates and O-methylated amines; the pooled organic phase was extracted once with one-sixth its volume of 1 M K_2CO_3 buffer (pH10). The buffer layer (containing the acids) was adjusted to pH1 (glass electrode) with concentrated HCl and extracted thrice, each time with double volume of ethyl acetate. To each combined ethyl acetate fraction 10 µg of unlabelled VMA and DHMA* were added, and the samples were

* DHMA - Dihydroxymandelic acid.

blown down to \approx 0.2 ml. under a stream of nitrogen, then stored at -20°C until chromatography.

(2) O-methylated Amines from Glycol Conjugates: Each aqueous phase was adjusted to pH7.5 (glass electrode) with 5N, 1N and 0.1N KOH, and passed over a 10 x 0.7 cm. column of Amberlite CG 50 resin (NH_4^+ form, pH7.5). The effluent was saved, the column was washed with 10 ml. of distilled water and the O-methylated amines were eluted off the column with 15 ml. of 3N NH_4OH . To each effluent (containing the glycol conjugates) and eluate \approx 200 μg of ascorbic acid was added; 10 μg of NM and M were added to the latter. They were then each reduced in volume to \approx 0.2 ml. under vacuum at 37°C and stored at -20°C until chromatography.

(C) IDENTIFICATION

The acids, glycol conjugates and O-methylated amines were identified using descending paper chromatography. All tanks were saturated with nitrogen before sealing and the chromatograms were left to equilibrate with the solvent vapours for eight hours before developing.

(1) Phenolic Acids: Each 0.2 ml. ethyl acetate concentrate was applied to a 4.6 x 4 cm. strip of Whatman No. 1 chromatography paper*; the sides of the tube were washed with 0.2 ml. of methanol which was also applied to the strip. Markers of authentic DHMA, VMA, DHPG and HMPG were applied onto separate strips. The sample and marker

* A sheet of Whatman No. 1 chromatography paper was cut (along its length) into 4 cm. wide strips separated from each other by 1 cm. wide gaps. The gaps did not reach the opposite edges of the paper; thus the strips were connected to each other.

strips were developed in Isobutyl methyl ketone:4% formic acid (10:1 v/v ; 2 phase) for six hours; then they were removed from the tank and dried.

The marker strips were sprayed with a mixture of paranitro-aniline:10% sodium nitrite (10:1), allowed to dry and resprayed with 1N NaOH in 50% ethanol. The visualized marker spots were localized and their distances from the origin measured (Figure 14,14a).

The sample strips were cut into 1 x 4 cm. strips, each of which was immersed in 4 ml. of Toluene (PPO, POPOP) scintillator and counted by liquid scintillation against blank strips developed in the same solvent system (Figure 14,14a).

(2) Glycol Conjugates: Each 0.2 ml. concentrated effluent was applied to a 46 x 4 cm. strip of Whatman No. 1 chromatography paper; the sides of the tube were washed with 0.2 ml. of methanol, which was also applied to the strip. Paranitrophenol was applied onto separate strips which, together with the sample strips, were developed in chloroform:methanol:ammonia (0.88) (12:7:1 v/v ; single phase) for thirteen hours, according to the method of R.F. Sugden (personal communication)*.

* The paranitrophenol gives a yellow colour in a basic medium, thus is a convenient marker during development of the chromatogram. At about thirteen hours it reaches the lower edge of the paper and that is the time to stop the chromatogram, since the HMPG conjugate runs just behind it. In that solvent system the glycol conjugates run slowest with the DHPG conjugate moving only a short distance from the origin; the HMPG conjugate runs further from the origin and therefore is separated from it. The chromatogram is developed for thirteen hours because by that time: (i) the free alcohols and phenolic acids run off the lower edge of the paper, (ii) the HMPG conjugate is well separated from the DHPG conjugate without the former running off the paper, and (iii) the DHPG conjugate is well separated from contaminants which remain at the origin.

The sample strips were removed from the tank, dried and cut into 1 x 4 cm. strips, each of which was immersed in 4 ml. of Toluene (PPO, POPOP) scintillator. They were counted by liquid scintillation against blank strips developed in the same solvent system (Figure 15,15a).

(3) O-methylated Amines: Each 0.2 ml. concentrated eluate was applied to a 46 x 4 cm. strip of Whatman No. 1 chromatography paper; the sides of the tube were washed with 0.2 ml. of methanol which was also applied to the strip. Markers of authentic NM, M and NA were applied onto separate strips which, together with the sample strips, were developed in butanol:ethanol:ammonia (0.88) (4:1:1 ^v/_v - single phase) for seventeen hours. At the end of that time they were removed from the tank and dried -

1. The marker strips were sprayed with a mixture of paranitroaniline: 10% sodium nitrite (10:1), dried and resprayed with ethanolic 1N NaOH. The visualized spots were localized and their distances from the origin were measured (Figure 16,16a).

2. The sample strips were cut into 1 x 4 cm. strips, each of which was immersed in 4 ml. of Toluene (PPO, POPOP) scintillator and counted by liquid scintillation against blank strips developed in the same solvent system (Figure 16,16a).

RESULTS

1. All values were corrected for (i) quenching by the channel ratio method; thus expressed as DPM, and (ii) recoveries, except the conjugates.

2. In the case of the glycol conjugates and acids, the ratio of the O-methylated/dihydroxy catabolite was calculated.

3. In all three (acids, glycol conjugates and O-methylated amines), the value of each catabolite in any one sample was expressed as -

(i) Per cent of total radioactivity in the supernatant.

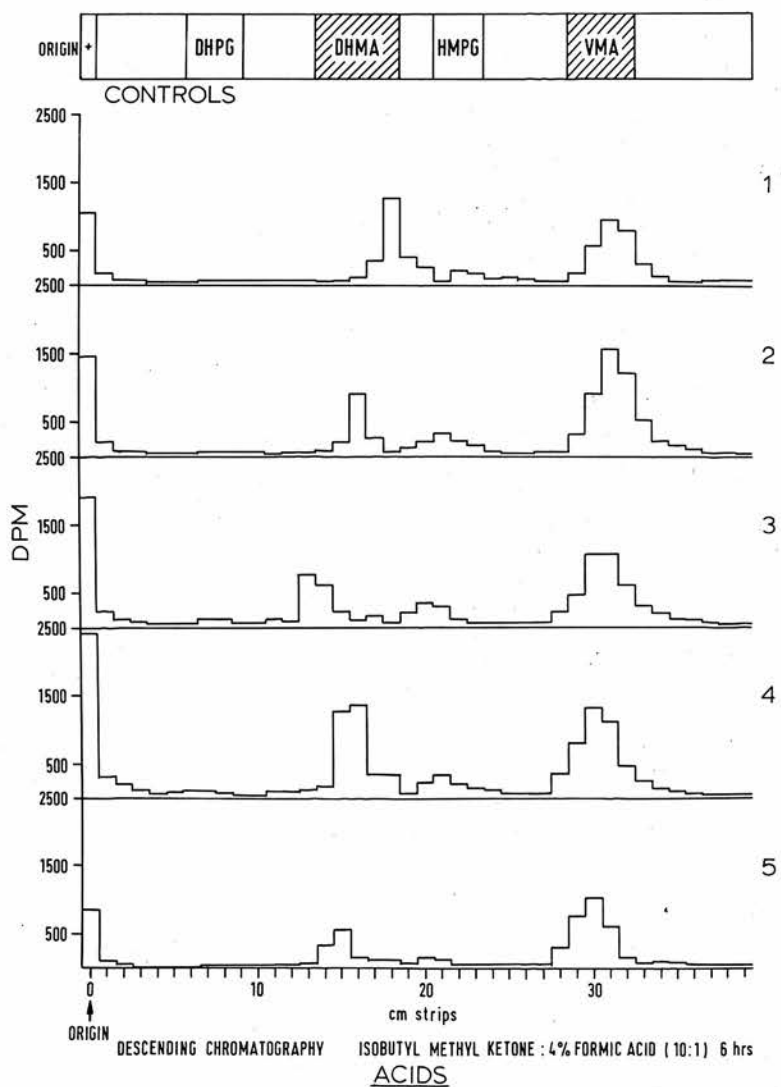
(ii) Per cent of total catabolites obtained from adding the individual peaks of radioactivity from all the catabolites, e.g. -

$$\frac{\text{DHMA (in DPM)}}{(\text{DHMA} + \text{VMA}) + (\text{DHPG} + \text{HMPG conjugates}) + \text{NM (in DPM)}} \times 100$$

(1) Phenolic Acids

As seen in Figures 14 and 14a, there was some carry over of free (unconjugated) HMPG and a lesser amount of free DHPG. However, they were well separated from DHMA.

FIGURE 14.

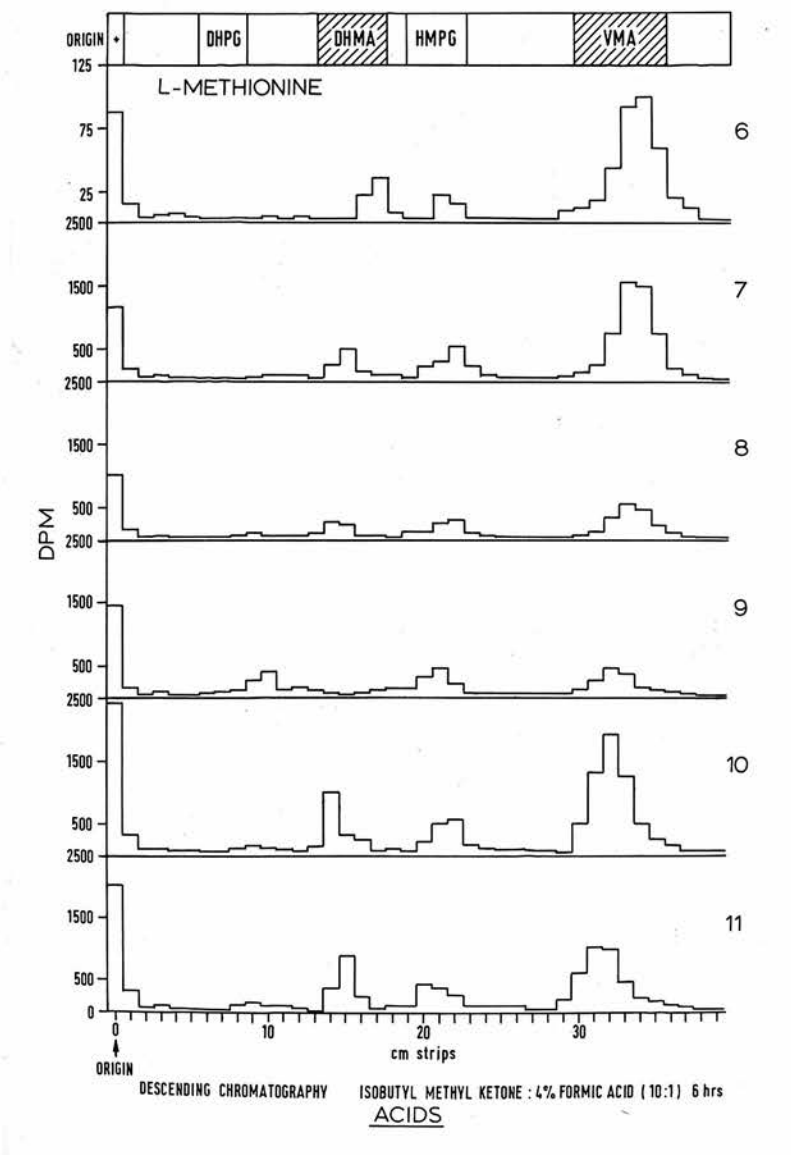


ACID CATABOLITES OF C¹⁴-NA IN RAT BRAIN
ONE HOUR AFTER ITS INTRAVENTRICULAR INJECTION
(Control (glycine) group)

Whatman No. 1 chromatography paper strips (46 x 4 cm.).

Markers were visualized by spraying with diazotised
paranitroaniline, then ethanolic 1N NaOH.

FIGURE 14a



ACID CATABOLITES OF C¹⁴-NA IN RAT BRAIN
ONE HOUR AFTER ITS INTRAVENTRICULAR INJECTION
(L-Methionine group)

Whatman No. 1 chromatography paper strips (46 x 4 cm.)

Markers were visualized by spraying with diazotised
paranitroaniline, then ethanolic 1N NaOH.

TABLE 10

RATIO OF VMA/DHMA IN BRAIN ONE HOUR AFTER
INTRAVENTRICULAR INJECTION OF C¹⁴-NA

CONTROL*		L-METHIONINE**	
1	1.4132	6	5.3064
2	2.3329	7	5.5447
3	2.3464	8	3.7866
4	1.3101	9	4.2791
5	2.2571	10	4.0280
		11	2.6101

* Group of rats which were injected intraperitoneally with glycine.

** Group of rats which were fed and injected intraperitoneally with L-methionine.

The t-test for uncorrelated means (Guilford, 1956) showed that t (d.f. 9) = 3.9739 ($t > 3.250$ significant at 0.01 level).

TABLE 10a

DHMA AND VMA PER CENT OF TOTAL RADIOACTIVITY IN SUPERNATANT
OF BRAIN ONE HOUR AFTER C¹⁴-NA INTRAVENTRICULAR INJECTION

	CONTROL			L-METHIONINE	
	DHMA	VMA		DHMA	VMA
1	1.17	1.5	6	0.24	1.31
2	0.79	1.86	7	0.38	2.15
3	0.70	1.65	8	0.35	1.34
4	0.89	1.17	9	0.18	0.57
5	0.67	1.53	10	0.48	1.94
			11	0.74	1.93

DHMA - The t-test for uncorrelated means showed that
 t (d.f. 9) = 3.2927 ($t > 3.250$, significant at 0.01 level).

VMA - The Wilcoxon Rank Sum Test (Wilcoxon and Wilcox, 1964)
 showed that $T = 28$ (for significance $T < 20$ or > 40).

TABLE 10b

DHMA AND VMA PER CENT OF TOTAL CATABOLITES IN BRAIN
ONE HOUR AFTER C¹⁴-NA INTRAVENTRICULAR INJECTION

	CONTROL			L-METHIONINE	
	DHMA	VMA		DHMA	VMA
1	4.60	5.88	6	1.90	10.11
2	3.33	7.77	7	1.74	9.69
3	2.94	6.91	8	1.45	5.51
4	6.71	8.80	9	0.59	2.85
5	3.74	8.44	10	2.19	8.82
			11	3.81	9.96

DHMA - The t-test for uncorrelated means showed that
t (d.f. 9) = 2.9986 (t > 2.262, significant at 0.05 level).

VMA - The Wilcoxon Rank Sum Test showed that T = 25
(for significance T < 20 or > 40).

Comment: The significant difference in VMA/DHMA ratios between the control and L-methionine groups was due to a change in DHMA and not VMA. The results showed a decrease of DHMA in the L-methionine group (Tables 10a and 10b), hence the increase in VMA/DHMA ratio (Table 10).

- (2) Glycol Conjugates (HMPG SO₄ and DHPG SO₄)
(Figures 15 and 15a).

TABLE 11

RATIO OF HMPG SO₄/DHPG SO₄ IN BRAIN
AFTER INTRAVENTRICULAR INJECTION OF C¹⁴-NA
(Time interval - 1 hour)

CONTROL		L-METHIONINE	
1	2.1545	6	2.8555
2	1.5926	7	2.2615
3	1.3707	8	1.4568
4	1.3803	9	1.3111
5	1.8988	10	1.4321
		11	1.4884

The Wilcoxon Rank Sum Test showed that $T = 29$
(for significance $T < 20$ or > 40).

TABLE 11a

DHPG SO₄ AND HMPG SO₄ PER CENT OF TOTAL RADIOACTIVITY IN SUPER-
NATANT OF BRAIN ONE HOUR AFTER C¹⁴-NA INTRAVENTRICULAR INJECTION

	CONTROL			L-METHIONINE	
	DHPG SO ₄	HMPG SO ₄		DHPG SO ₄	HMPG SO ₄
1	6.62	14.27	6	2.52	7.20
2	7.09	11.58	7	5.38	12.16
3	6.99	9.81	8	7.65	11.15
4	3.68	5.08	9	6.09	7.98
5	4.81	9.13	10	6.72	9.62
			11	5.31	7.90

DHPG SO₄ - The Wilcoxon Rank Sum Test showed that T = 31.

HMPG SO₄ - The Wilcoxon Rank Sum Test showed that T = 33

(for significance T <20 or >40).

TABLE 11b

DHPG SO₄ AND HMPG SO₄ PER CENT OF TOTAL CATABOLITES
IN BRAIN ONE HOUR AFTER C¹⁴-NA INTRAVENTRICULAR INJECTION

	CONTROL			L-METHIONINE	
	DHPG SO ₄	HMPG SO ₄		DHPG SO ₄	HMPG SO ₄
1	25.91	55.82	6	19.37	55.33
2	29.57	48.30	7	24.16	54.64
3	29.26	41.02	8	31.27	45.56
4	27.51	37.97	9	30.09	39.45
5	26.50	50.33	10	30.51	43.70
			11	27.37	40.74

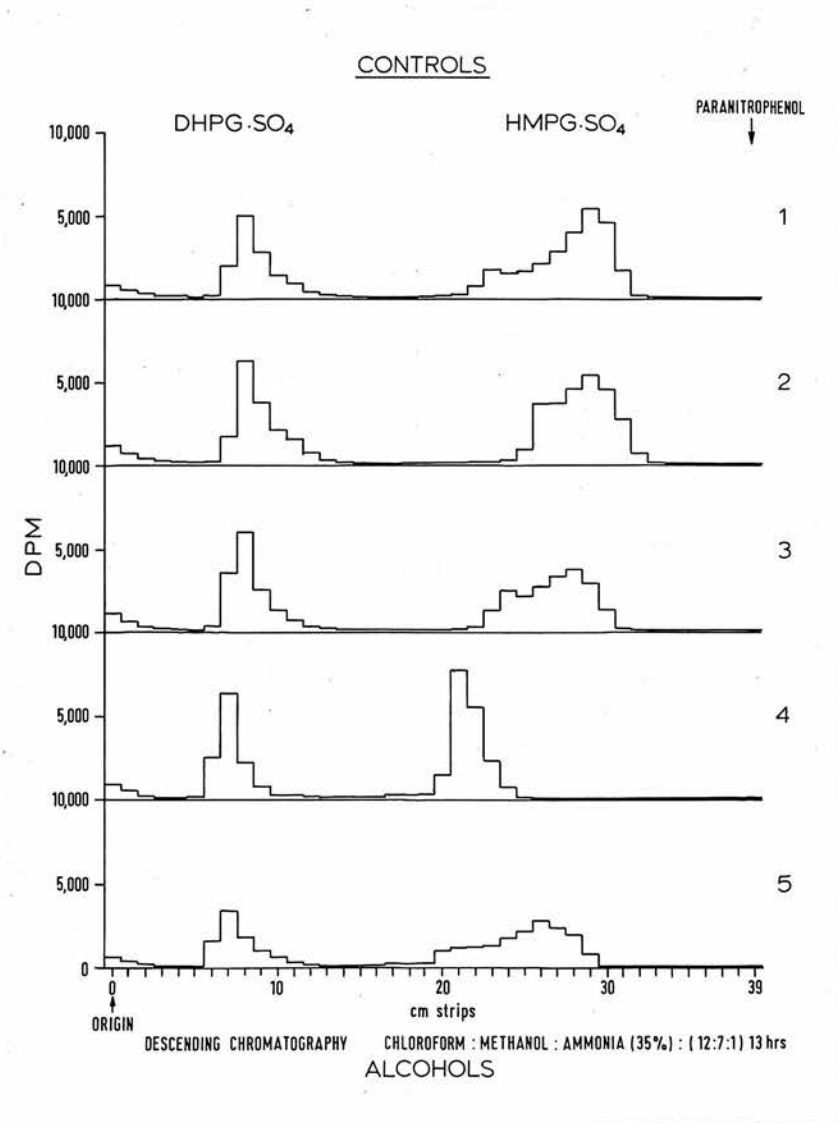
DHPG SO₄ - The Wilcoxon Rank Sum Test showed that T = 28.

HMPG SO₄ - The Wilcoxon Rank Sum Test showed that T = 31

(for significance T <20 or >40).

Comment: There was no change in either ratio (Table 11) or percentage of the glycols (Tables 11a and 11b) between the two groups, indicating that L-methionine did not alter these catabolites.

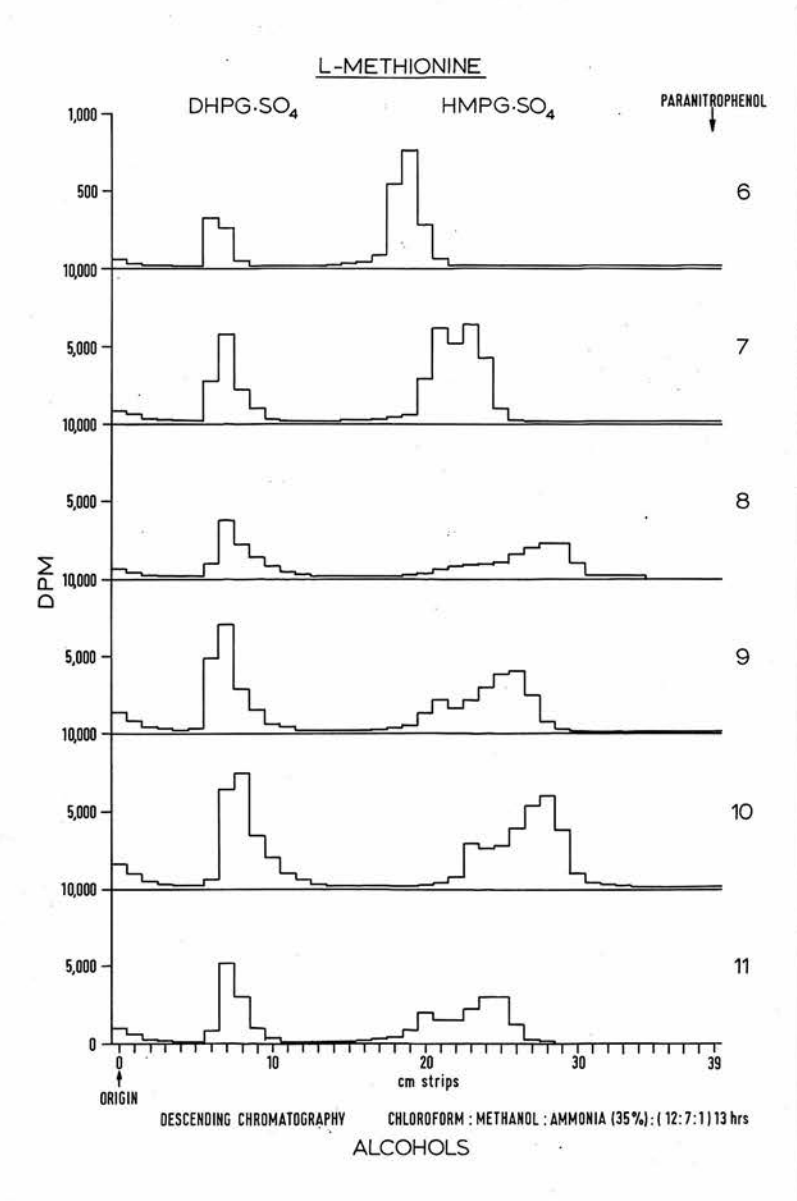
FIGURE 15



CONJUGATED GLYCOL CATABOLITES OF C¹⁴-NA IN RAT BRAIN
ONE HOUR AFTER ITS INTRAVENTRICULAR INJECTION
(Control (glycine) group)

Whatman No. 1 chromatography paper strips (46 x 4 cm.)

FIGURE 15a



CONJUGATED GLYCOL CATABOLITES OF C¹⁴-NA IN RAT BRAIN
ONE HOUR AFTER ITS INTRAVENTRICULAR INJECTION
(L-Methionine group)

Whatman No. 1 chromatography paper strips (46 x 4 cm.)

(3) O-methylated Amines

As seen in Figures 16 and 16a there were no peaks of radioactivity corresponding to M, which accounted for an insignificant percentage of the total catabolites.

TABLE 12

NM PER CENT OF TOTAL RADIOACTIVITY IN SUPERNATANT
OF BRAIN ONE HOUR AFTER INTRAVENTRICULAR INJECTION OF C¹⁴-NA

	NM		NM
	CONTROL		L-METHIONINE
1	1.98	6	1.72
2	2.64	7	2.17
3	4.74	8	3.96
4	2.54	9	5.46
5	1.99	10	3.25
		11	3.51

The Wilcoxon Rank Sum Test showed that $T = 26$
(for significance $T < 20$ or > 40).

TABLE 12a

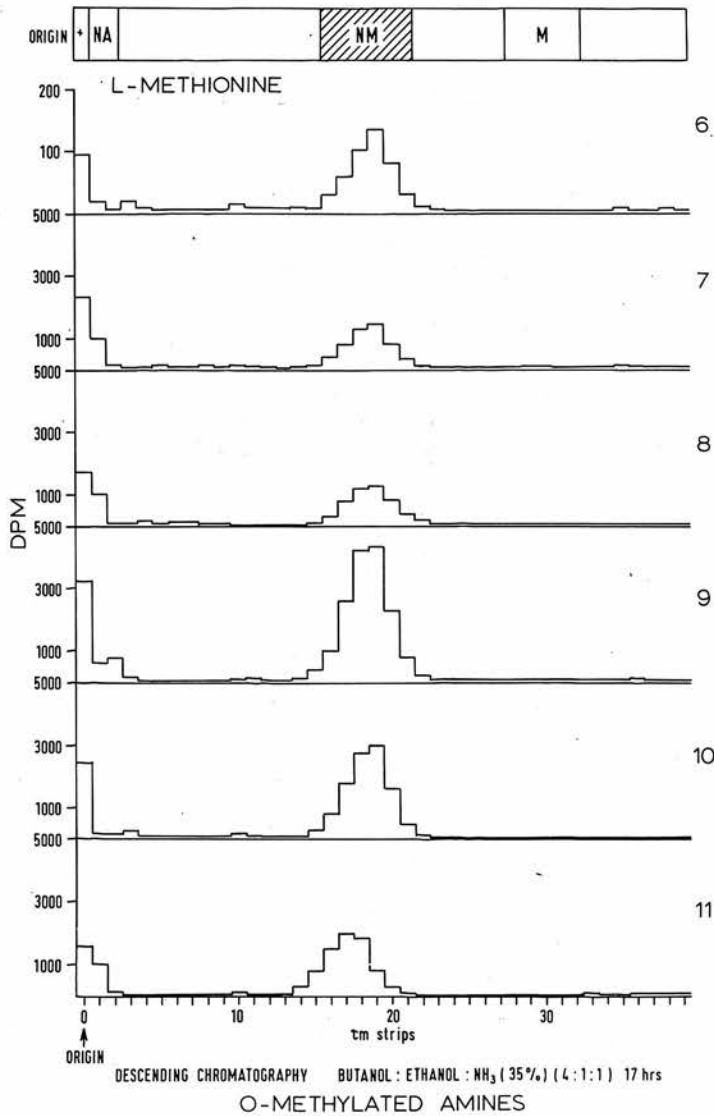
NM PER CENT OF TOTAL CATABOLITES IN BRAIN
AFTER C¹⁴-NA INTRAVENTRICULAR INJECTION

	CONTROL		L-METHIONINE
	NM		NM
1	7.7	6	13.25
2	11.00	7	9.74
3	19.85	8	16.19
4	18.99	9	27.00
5	10.97	10	14.76
		11	18.09

The Wilcoxon Rank Sum Test showed that $T = 27$
(for significance $T < 20$ or > 40).

Comment: L-methionine did not alter NM formation (Tables 12 and 12a).

FIGURE 16a



NORMETANEPHRINE IN RAT BRAIN ONE HOUR AFTER
INTRAVENTRICULAR INJECTION OF C¹⁴-NA
(L-Methionine group)

Whatman No. 1 chromatography paper strips (46 x 4 cm.)

Markers were visualized by spraying with diazotised
paranitroaniline, then ethanolic 1N NaOH.

TABLE 13

INTRA GROUP MEAN PER CENT OF TOTAL CATABOLITES
IN BRAIN AFTER C¹⁴-NA INTRAVENTRICULAR INJECTION
 (Time interval - 1 hour)

CONTROL					L-METHIONINE				
NM	HMPG SO ₄ (Conju- gate)	DHPG SO ₄ (Conju- gate)	VMA	DHMA	NM	HMPG SO ₄ (Conju- gate)	DHPG SO ₄ (Conju- gate)	VMA	DHMA
<u>13.70</u>	<u>46.69</u>	<u>27.75</u>	<u>7.56</u>	<u>4.26</u>	<u>16.50</u>	<u>46.57</u>	<u>27.15</u>	<u>7.82</u>	<u>1.95</u>
<u>±2.41</u> (SEM)	<u>±3.22</u> (SEM)	<u>±0.73</u> (SEM)	<u>±0.53</u> (SEM)	<u>±0.67</u> (SEM)	<u>±2.40</u> (SEM)	<u>±2.80</u> (SEM)	<u>±1.89</u> (SEM)	<u>±1.22</u> (SEM)	<u>±0.43</u> (SEM)

TABLE 14

INTRA GROUP MEAN PER CENT OF TOTAL RADIOACTIVITY IN SUPERNATANT
OF BRAIN ONE HOUR AFTER C¹⁴-NA INTRAVENTRICULAR INJECTION

CONTROL					L-METHIONINE				
NM	HMPG SO ₄ (Conju- gate)	DHPG SO ₄ (Conju- gate)	VMA	DHMA	NM	HMPG SO ₄ (Conju- gate)	DHPG SO ₄ (Conju- gate)	VMA	DHMA
<u>2.78</u>	<u>9.97</u>	<u>5.84</u>	<u>1.54</u>	<u>0.844</u>	<u>3.35</u>	<u>9.34</u>	<u>5.61</u>	<u>1.54</u>	<u>0.395</u>
<u>±0.50</u> (SEM)	<u>±1.51</u> (SEM)	<u>±0.68</u> (SEM)	<u>±0.11</u> (SEM)	<u>±0.09</u> (SEM)	<u>±0.54</u> (SEM)	<u>±0.81</u> (SEM)	<u>±0.71</u> (SEM)	<u>±0.24</u> (SEM)	<u>±0.08</u> (SEM)

DISCUSSION

The object of the experiments was to determine whether changes in O-methylation of NA could be demonstrated in animals given high doses of methionine over a long period. Mackenzie et al (1950) showed that there was an increase in turnover of methyl groups in the body following L-methionine feeding. This was investigated by feeding rats small doses of C¹⁴-methyl labelled L-methionine with or without L-methionine loading and measuring the radioactivity excreted in breath, urine and faeces. Their results showed that in the L-methionine loaded rats maximum turnover, thus possible utilization, of methyl groups occurred three hours after the administration of the labelled L-methionine and was sustained till six hours. Following that period there was a gradual decrease in turnover, reaching a plateau by twelve hours.

In this experiment the group of rats fed with L-methionine for four days were injected with C¹⁴-NA three hours following the intraperitoneal injection of L-methionine. According to the above findings, this time interval represents maximum availability of methyl groups for transmethylation. However, the present results do not show evidence of increased O-methylation of NA catabolites, which suggests that, although there is an increase in tissue levels of S-adenosylmethionine (active methyl group) on L-methionine loading (Baldessarini, 1967), either (i) there is no increase in methylation. This could perhaps be due to a reaction rate limiting factor which protects the body against non-physiological conditions; such limiting factors are known to exist in various metabolic reactions

in the body (e.g. active absorption of iron from the intestinal mucosa is limited by catalytic conversion of the ferric ion). Indeed, the limiting factor could be the methyl transferase (i.e. COMT) itself, which, like other enzyme systems, can be inhibited in the presence of excess co-enzyme (i.e. methyl donor) (Meister, 1965), or (ii) possibly an increase in methylation rate does occur but in turn leads to excess demethylation; the latter acts as a protective mechanism.

Regardless of the mechanism in which the body protects itself against non-physiological transmethylation, it is interesting to note that at one interval in time homeostasis was maintained where neither an increase in the O-methylated NA catabolites nor an increase in the dihydroxy catabolites (suggestive of increased demethylation) occurred. The decrease in DHMA on L-methionine loading is probably not indicative of increased methylation for the following reasons:-

(i) There was no concomitant decrease in DHPG (another dihydroxy catabolite) or a significant increase in NM to assume that this was due to a shift in the degradation of NA in favour of O-methylation and at the expense of deamination.

(ii) There was no accompanying increase in VMA. Although it is claimed that COMT is probably also found intraneuronally (Weiner, 1970 - personal communication with Iverson and Jarrott), it is believed that intraneuronal NA is deaminated by MAO forming the dihydroxy catabolites which then are transported from the cell and are O-methylated by extraneuronal COMT (Carlsson and Hillarp, 1962;

Kopin and Gordon, 1962). If the decrease in DHMA was due to increased O-methylation, then it is expected that VMA should also have increased unless such absolute increase was not shown because VMA is eliminated from brain and body at a rapid rate (Maas and Landis, 1968).

The mean values shown in Table 13, except for DHMA in the control group, are in agreement with those reported by Sugden, R. (1970 - personal communication). He studied the catabolism of C^{14} -NA in brain at various time intervals after its intraventricular injection. He showed that after one hour the values of the various catabolites, expressed as per cent of total catabolites, were as follows: 20% NM, 38% HMPG conjugate, 22% DHPG conjugate, 6% VMA and 1.5% DHMA. The higher percentage of DHMA in the control group probably represents wide variations in the formation and/or isolation of this catabolite (C. Yates - unpublished observations). However, there is the remote possibility that this could be a glycine effect. It is very difficult to interpret this variation in DHMA since the Sugden experiment represents control conditions, yet the DHMA value is similar to that in the L-methionine group and lower than that in the control group. Although this might suggest that there was no decrease in DHMA on L-methionine loading but only an experimental variation, it is interesting to note that the individual DHMA values within each group show that the control values are invariably higher (Tables 10a and 10b).

There was no statistically significant increase in the O-methylated catabolites of NA on L-methionine loading. This possibly

suggests, provided O-methylation is representative of other types of methylation (i.e. N-methylation, etc.), that in the body there is a protective mechanism which regulates transmethylation according to its needs.

CONCLUDING DISCUSSION

The present study has shown that L-methionine (without MAOI) can produce an acute psychosis in a proportion of chronic schizophrenics; these "reactors" cannot be differentiated from "non-reactors" on clinical grounds. The "methionine reaction" is schizophreniform in nature and is sometimes preceded by a toxic reaction. Although it takes the form of a schizophrenic psychosis and sometimes even exacerbates specific and latent symptoms, it cannot be considered for certain to be a specific exacerbation of the schizophrenic illness; it could very well be a non-specific superimposed psychosis produced by L-methionine, which may take this form in schizophrenics. However, comparable (Alexander et al, 1963) or lower (Harper et al, 1970) dosages of L-methionine do not produce a similar reaction in non-schizophrenic patients or normals respectively.

Following the immediate reaction to L-methionine there was no significant improvement in the mental state of any of the patients; however, none became worse. It may be concluded, therefore, that L-methionine is without therapeutic effect.

The mechanism by which L-methionine produces this reaction is quite unknown. The following points are, however, relevant.

(1) L-methionine has been shown to be the prime methyl donor in the body (Cantoni, 1951b) and to donate methyl groups to brain monoamines in vitro (Axelrod et al, 1958) and most probably in vivo (the present study - Section IV). (2) There was no increase in O-methylation of catechol amines on L-methionine loading, as

reflected by the absence of an increase in (a) HMPG and VMA in the urine of schizophrenics, and (b) the O-methylated noradrenaline catabolites in rat brain. However, the possibility that, in schizophrenia, there is a "transmethylation defect" and that L-methionine (being a methyl donor) produces its effects through this mechanism cannot be completely ruled out. This is because, firstly, only the O-methylation of catechol amines was studied, thus L-methionine, being a methyl donor for other compounds in the body (i.e. N and O-methylation of indole amines), could have exerted its effect through these compounds. As mentioned in the literature review, little is known about these methylating enzymes and many O and N-methylated derivatives of indole amines are psychotomimetic. Secondly, the defect in schizophrenia could very well be that of demethylation. Such a defect would result in an increased body methylation pool, on L-methionine loading, without an absolute increase in the rate of methylation.

Moreover, L-methionine has functions in the body other than that of methyl donation. Such functions include thiol group activity, interaction with tryptophane metabolism, cell membrane transport, etc. (See literature review). It is quite possible, therefore, that its effects could have been mediated through any of these functions which could be unrelated to the pathogenesis of schizophrenia. On the other hand, abnormal methionine metabolism (which includes the above functions) might be involved in schizophrenia (Israelstam et al, 1970; Spiro et al, 1965).

This study has suggested further investigations into biological transmethylation and its enzyme systems in animals as well as humans, both schizophrenics and normals. Specifically, (i) the confirmation of relevant previous work and the study of specificity and nature of methylating and demethylating enzymes; (ii) investigation of indole amines and their catabolites in normals and schizophrenics; (iii) a study of methionine metabolism in schizophrenics; and (iv) a further longitudinal study of HMPG urinary levels in a larger group of schizophrenics and normals, and the effect of L-methionine loading on these levels in the latter group.

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APPENDIX I

PSYCHIATRIC HISTORIES

(A) PILOT1. Age: 52 yearsHospital Diagnosis: SchizophreniaDuration of Hospitalization: 30 years

The patient was apathetic and withdrawn. At times she showed manneristic behaviour and auditory hallucinations. In the ward she was tidy and aware of her environment. She was on Largactil, 50 mgm at bedtime. When the medication was stopped she did not show any change in her mental state.

On the second day of drug A (found later to be L-methionine) she became more withdrawn and disinterested in her surroundings. At times she became restless and showed severe thought blocking. The auditory hallucinations became more frequent and were suspected to influence her behaviour in the ward. The manneristic movements became more pronounced. There were no signs or symptoms of clouding of consciousness or disorientation. This condition remained until L-methionine was withdrawn when her mental state reverted to that of the pre-L-methionine period. She became more sociable and responsive. The hallucinations became less frequent and, in the ward, she showed more interest in other patients and the nursing staff. Phentiazines were not restarted and the long term follow-up for one year did not show any significant changes in her mental state.

*7 has long
before recovery
- related
mental pattern*

2. Age: 58 yearsHospital Diagnosis: SchizophreniaDuration of Hospitalization: 24 years

Before the beginning of the trial, this patient was sociable and active in the ward. She showed thought dissociation and bizarre

thought content. At times she manifested with outbursts of impulsive behaviour and paranoid feelings against the nursing staff. She admitted hearing voices that instructed her to do certain things, which she did not divulge. Her affect was incongruous. She was not receiving any medication.

On the third day of drug B (later found to be L-methionine) she became very deluded, showing continuous signs of persecutory thoughts. She became antagonistic to everybody and, at times, showed aggressive behaviour. The auditory hallucinations became more marked and frequent and the patient could be observed talking to herself. The patient's affect was blunt. She did not seem to be disorientated and did not show signs of clouding of consciousness. When L-methionine was stopped she gradually improved by becoming friendlier, sociable and communicative. The auditory hallucinations became less frequent and the patient had partial insight into the episode. The long term follow-up did not show a significant change in her mental state.

3. Age: 61 years

Hospital Diagnosis: Chronic schizophrenia

Duration of Hospitalization: 35 years

This patient was withdrawn unless approached. She responded to her environment but showed no stamina or interest to communicate with others. She was negativistic but, when talked to, betrayed thought form and content disorder. She denied being hallucinated and did not show outward delusions; her affect was flat. She was on no medications.

There was no observable change in her behaviour throughout the experiment and she did not show signs of a toxic delirium.

Long term follow-up was uneventful.

4. Age: 54 years

Hospital Diagnosis: Paranoid schizophrenia

Duration of Hospitalization: 16 years

This patient was sociable and communicative. She admitted having auditory hallucinations (mainly persecutory) at night. She got along with everybody and did not show signs of antagonism or hatred to anybody. She was keen on participating in social events and was reported to be helpful in the ward. Her affect was rather flat. No reaction to L-methionine was observed, and the patient's long term follow-up was uneventful. She was not receiving drugs before or after the experiment.

No effect of
no affect.

5. Age: 46 years

Hospital Diagnosis: Heberphrenic schizophrenia

Duration of Hospitalization: 15 years

This patient showed frequent manneristic behaviour and incongruity of affect. She admitted experiencing auditory hallucinations but it was very difficult to assess her thought content and form because of her short attentive span. In the ward she was active but not very helpful. L-methionine did not produce any worsening of her symptoms.

She was on Fentazin 4 mgm b.i.d. and her mental state did not deteriorate after stopping it. After the experiment, only a night sedative was given and the long term follow-up showed little change in her mental state.

(B) MAIN EXPERIMENT
(1-5 female; 6-11 male)

1. Age: 60 years

Hospital Diagnosis: Heberphrenic schizophrenia

Duration of Hospitalization: 25 years

At the start of the experiment she was sociable, orientated and showed minimal schizophrenic symptoms. These symptoms comprised of thought form disorder as well as those of thought content. The latter were in the way of persecutory delusions and auditory hallucinations, which were intermittent. At times she showed withdrawal and negativism along with suspiciousness. These symptom fluctuations were never severe and subsided within a day or two; on few occasions they were triggered by environmental factors. She was on Stelazine 5 mgm b.i.d. and the clinical picture before the start of the experiment showed more sociability and less apathy and withdrawal following discontinuation of the phenothiazines.

She reacted to L-methionine (amino acid B) firstly by a toxic psychosis, which was characterized by disorientation and altered sensorium, accompanied by neurological signs of vestibular dysfunction (ataxia, Romberg sign). This reaction subsided after 24-48 hours and was followed by a symptom free period lasting 12-14 hours. The second reaction was schizophreniform in nature and was free of any toxic signs or symptoms. The patient became impulsive and very suspicious. Her ward behaviour indicated that she felt persecuted by the staff and other patients. She was agitated and restless with continuous auditory hallucinations which in content were similar to those experienced by the patient some years ago. Her affect became blunt and she became negativistic.

Her mental state was considered acute and it was felt that the amino acid (later found to be L-methionine) should be withdrawn. On the eighth day of the L-methionine period, one day after L-methionine was stopped, the acute symptoms started becoming less severe and on the fourteenth day she settled down. She was reported by the nursing staff to be more sociable and less suspicious than before the experiment started; the delusions and hallucinations were less apparent clinically. However, this improvement did not last and the patient showed a minor relapse. Stelazine (5 mgm t.i.d.) was started on the seventeenth day and the patient responded well to it. The long term follow-up proved uneventful and the patient maintained a mental state similar to that before the experiment.

2. Age: 46 years

Hospital Diagnosis: Heberphrenic schizophrenia

Duration of Hospitalization: 10 years

Before the experiment the patient showed incongruous affect, thought blocking and moderate thought form disorder. She was manageable but unsociable and at times experienced auditory hallucinations. She was on Melleril (50 mgm bi.d.) and showed no relapse after its discontinuation. She reacted to L-methionine by worsening of the above symptoms, which subsided on its withdrawal two weeks later. Specific hallucinations and delusions pertaining to the same situations and persons which she had some years ago were experienced by the patient. At the end of the experiment she was restored on phenothiazines (Melleril 50 mgm b.i.d.), to which she responded. Her affect became less incongruous and the hallucinations were less frequent. In the ward she was reported to be more

sociable and communicative than she had been before the experiment started. The long term follow-up showed that these changes persisted.

3. Age: 59 years

Hospital Diagnosis: Schizophrenia

Duration of Hospitalization: 29 years

This patient was withdrawn and asociable before the experiment. At times she was hallucinated, showed thought blocking, and her affect was flat. She was not receiving any medications. She did not react to L-methionine and at the end of her stay in the metabolic ward she was reported by the nursing staff to be more manageable, sociable and attentive than she had been before. She was not put on any drugs and kept well after transfer to her ward.

4. Age: 55 years

Hospital Diagnosis: Schizophrenia

Duration of Hospitalization: 37 years

Before the experiment she was apathetic, withdrawn, but at times showed impulsive behaviour. She was suspected to be hallucinated but the patient was negativistic. Her affect was flat and she did not show any fluctuations of her symptoms. She was not receiving medications.

She did not react to L-methionine and there was no change in her behaviour or clinical state throughout the experiment. She was not put on drugs at the end of the experiment, and the long term follow-up was uneventful.

5. Age: 57 yearsHospital Diagnosis: SchizophreniaDuration of Hospitalization: 25 years

This patient was sociable in the ward, helpful and interested in her environment. At times she showed paranoid behaviour and became withdrawn. Auditory hallucinations were not noticed at any time and the patient denied experiencing them. She was not on any drugs before the experiment. The natural history of her illness was stable, without fluctuations for the past four to five years.

She reacted to L-methionine in that she became negativistic, withdrawn, suspicious and developed persecutory delusions. She was overtly hallucinated and her behaviour was suspected to be influenced by the auditory hallucinations. There were no signs or symptoms of a toxic state.

All the symptoms subsided three days after L-methionine was discontinued. She was not put on medications and, at the end of the experiment, she showed no significant changes in behaviour as compared to previous ratings. Long term follow-up was uneventful.

6. Age: 51 yearsHospital Diagnosis: Heberphrenic schizophreniaDuration of Hospitalization: 20 years

This patient reacted to L-methionine with acute schizophreniform symptoms and, prior to the experiment, discontinuation of the medication (Haloperidal 5 mgm b.i.d.) did not result in a relapse. At the start of the experiment he was communicative, interested in his environment, but some times showed paranoid behaviour. No

auditory hallucinations were reported and his affect was rather blunt. Before the start of Haloperidal (which he received for two years) he was more withdrawn and some times acted upon his paranoid feelings. He was not hallucinated. The reaction to L-methionine (amino acid A) took the form of auditory hallucinations (mainly with persecutory overtones), paranoid delusions, withdrawal, thought block and lack of concentration. There was no clinical evidence to suggest a toxic psychosis. Ward behaviour and the nurses' rating confirmed the above findings. These symptoms subsided gradually on stopping L-methionine. Haloperidal (5 mgm b.i.d.) was restarted at the end of the experiment and he was reported to be sociable, free of apparent symptoms and more communicative than he was before the experiment. Long term follow-up showed that he maintained an improved mental state.

7. Age: 53 years

Hospital Diagnosis: Catatonic schizophrenia

Duration of Hospitalization: 16 years

Prior to the experiment he showed lack of concentration, manneristic behaviour and thought form disorder. Phenothiazines (Largactil 50 mgm b.i.d.) were stopped, giving no worsening of his mental condition. He reacted to L-methionine (amino acid B), firstly by a toxic psychosis where he became disorientated, wandering about aimlessly; his sensorium was dulled and he showed at times ataxic gait. This reaction lasted for a period of about 36 hours and was followed by a symptom free interval of 24 hours. The second reaction was schizophreniform in nature, without any toxic

symptoms or signs, and took the form of a worsening in his pre-methionine mental state. Due to the marked exacerbation of his symptoms, L-methionine was withdrawn after eight days. The patient became better, but Melleril 50 mgm t.i.d. was restarted on the fourteenth day (six days after stopping L-methionine) and he showed a good response to it. At the end of the experiment he was observed to be sociable in the ward, doing things for himself, and his behaviour was manageable. Long term follow-up showed that he was no better or worse at the end of the experiment than he was at the beginning.

8. Age: 55 years

Hospital Diagnosis: Paranoid schizophrenia

Duration of Hospitalization: 27 years

This patient showed minimal clinical symptoms other than intermittent overtalkativeness and suspiciousness. All through he admitted having auditory hallucinations which did not influence his behaviour in any way. He did not relapse on stopping the phenothiazines. He did not react to L-methionine and towards the last six weeks of the experiment he was slightly more suspicious, admitting at times persecutory delusions; his overtalkativeness was slightly more marked. He was restarted on phenothiazines (Fentazine 8 mgm t.i.d.) at the end of the experiment and the above symptoms subsided, except for the auditory hallucinations, which became less frequent. The patient died three months after the end of the experiment, due to intussusception and intestinal gangrene.

9. Age: 58 years

Hospital Diagnosis: Schizophrenia

Duration of Hospitalization: 36 years

The patient showed manneristic behaviour, apathy and flatness of affect. He was withdrawn but manageable in the ward, and only occasionally was suspected to experience auditory hallucinations. He was on Largactil 25 mgm t.i.d. and after stopping the drug he did not show a relapse.

He did not react to L-methionine and his clinical mental state was unchangeable except for minor changes in ward behaviour from day to day. He was not restarted on phenothiazines and his long term follow-up was uneventful.

10. Age: 51 years

Hospital Diagnosis: Paranoid schizophrenia

Duration of Hospitalization: 14 years

At the beginning of the experiment he showed manneristic behaviour, withdrawal and incongruity of affect. He was at times hallucinated and manifested thought block, lack of concentration and thought dissociation. These symptoms did not increase in severity after discontinuation of the phenothiazines (Melleril 50 mgm b.i.d.) but did so on L-methionine. After the withdrawal of L-methionine he gradually became better and at the end of the experiment was restarted on Melleril 50 mgm b.i.d., on which further improvement occurred. Long term follow-up showed that the patient's mental state was similar to that before the experiment.

11. Age: 49 years

Hospital Diagnosis: Catatonic schizophrenia

Duration of Hospitalization: 12 years

This patient showed catatonic behaviour, negativism, withdrawal and thought blocking. He manifested thought form disorder but denied experiencing auditory hallucinations, and was communicative. The above symptoms were present at the beginning of the experiment and became exaggerated on L-methionine. Auditory hallucinations became continuous and their content was the same as those experienced years ago by the patient. In fact, the same voices were heard giving the same commands and slogans to the patient.

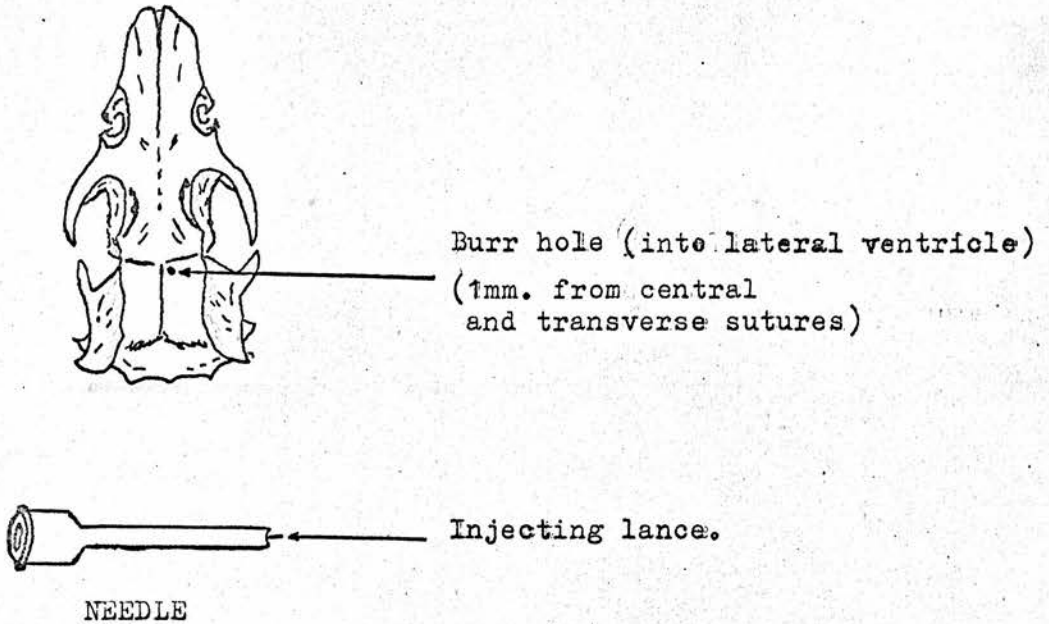
After stopping L-methionine he became gradually better and reverted to his pre-methionine mental state. He was not receiving any drugs prior to the start of the experiment and was not given any after it. The long term follow-up was uneventful.

APPENDIX II

DIAGRAM FOR INTRAVENTRICULAR INJECTION

APPENDIX II

DORSAL VIEW OF RAT SKULL



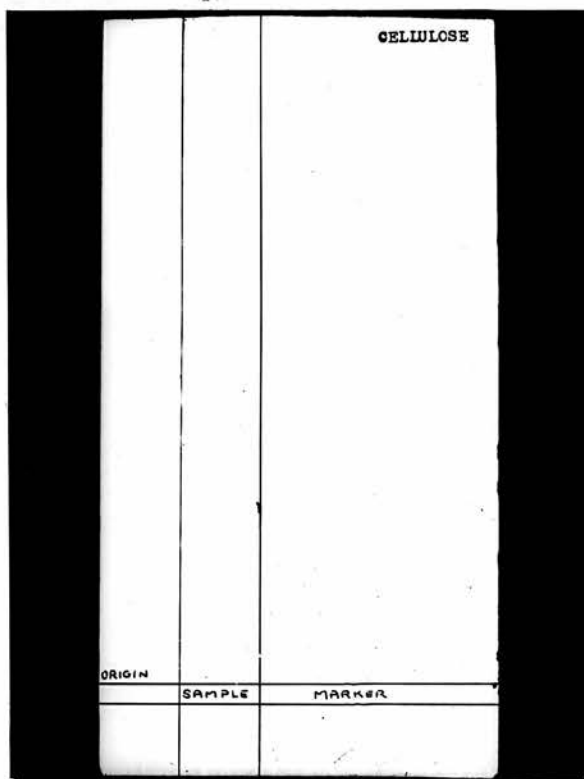
....DIAGRAM for INTRAVENTRICULAR INJECTION....

Procedure

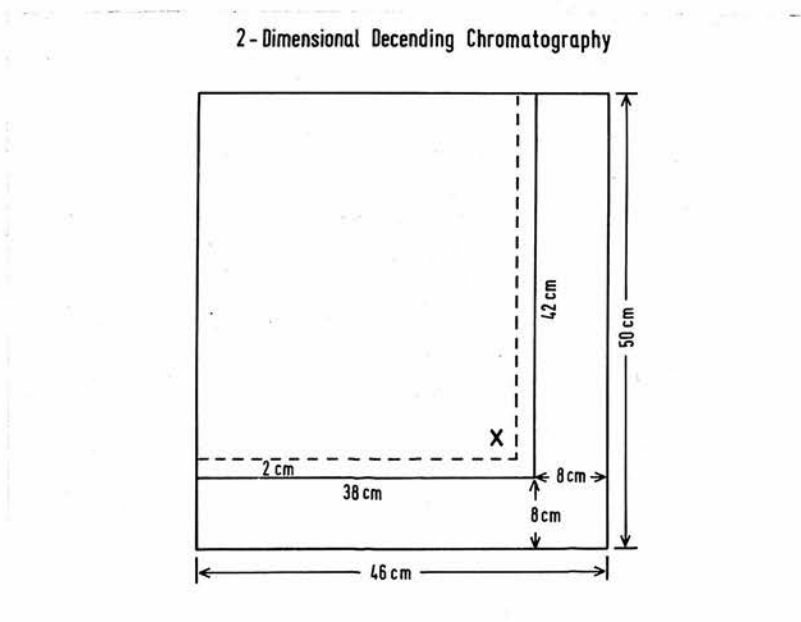
1. A 2-3 cm. midline incision is made on dorsum of skull.
2. A burr hole is made 1 mm. from the central and transverse sutures.
3. The injecting lance is inserted into the burr hole and the solution is injected.
4. The injecting lance is left in place for 15-20 seconds to avoid back flow of the solution.
5. The lance is removed and the incision edges are approximated with a Michael clip.

APPENDIX III

- (a) THIN LAYER CELLULOSE PLATE
- (b) WHATMAN NO. 1 CHROMATOGRAPHY PAPER



(a) Thin layer precoated cellulose plate (20 x 10 cm.)

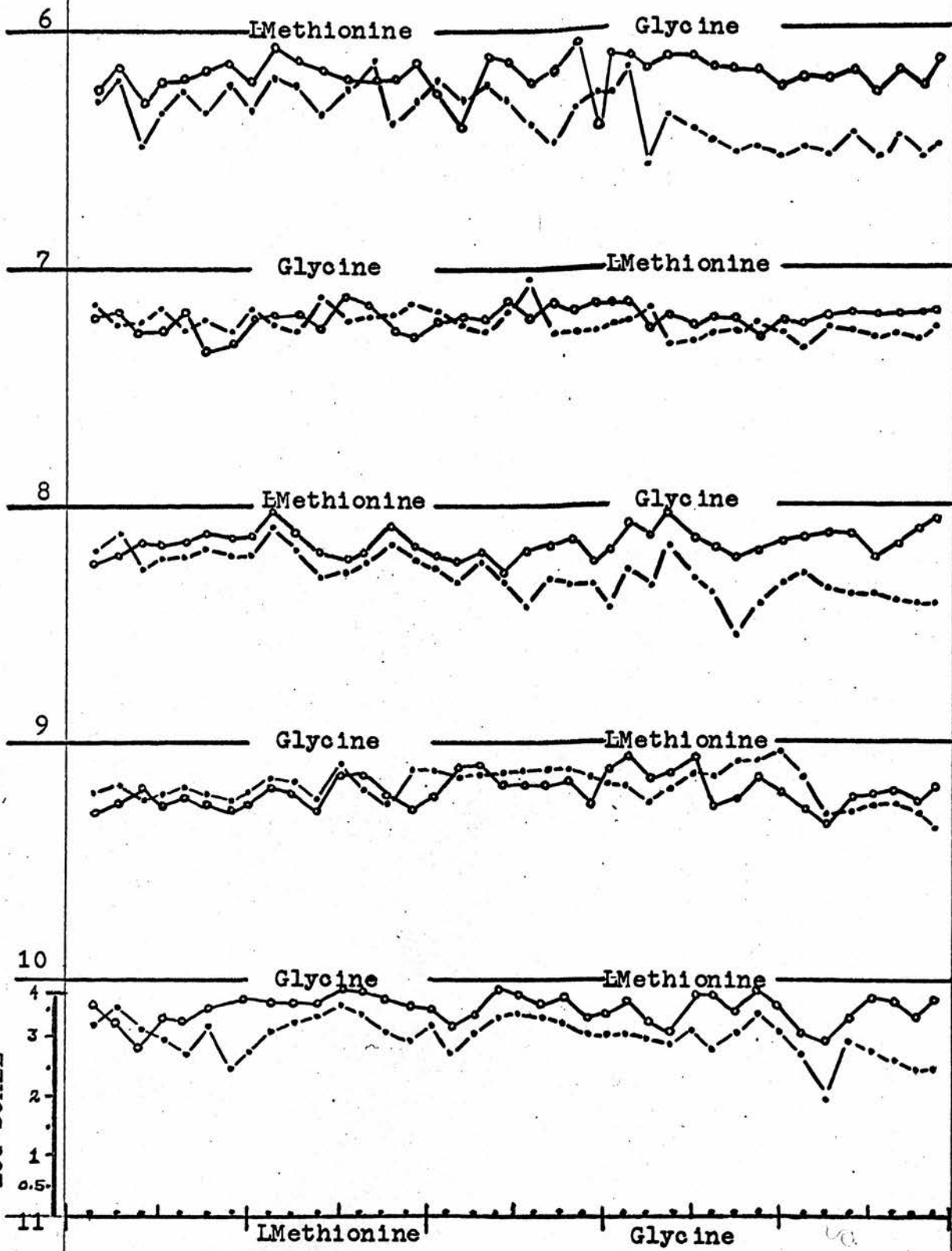


(b) Whatman No. 1 chromatography paper.

APPENDIX IV

HMPG AND VMA EXCRETION
(Main experiment - males)

PATIENT



APPENDIX
IV

LOG SCALE

MAIN EXPERIMENT

- Urine collection (Males)
- + 1 Week period
- + 2 Week period
- 24 hr. Urinary HMPG
- 24 hr. Urinary VMA

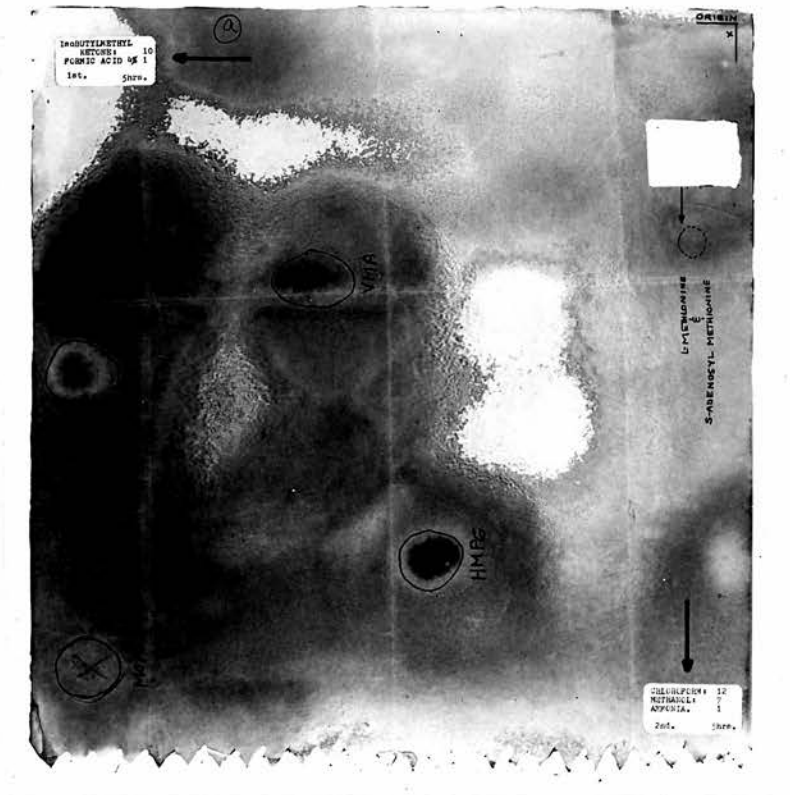
APPENDIX V

TWO DIMENSIONAL CHROMATOGRAPHY SYSTEM

1. Acids and Alcohols
2. O-Methylated Amines

Two Dimensional Descending Chromatography

(a)



1st System - Isobutyl methyl ketone:4% formic acid
(10:1 v/v) - 2 phase (organic phase used)
Developing time: 5 hours.

2nd System - Chloroform:methanol:ammonia (0.88)
(12:7:1 v/v) - single phase
Developing time: 5 hours.

a,b,c:

Whatman No. 1 chromatography paper.

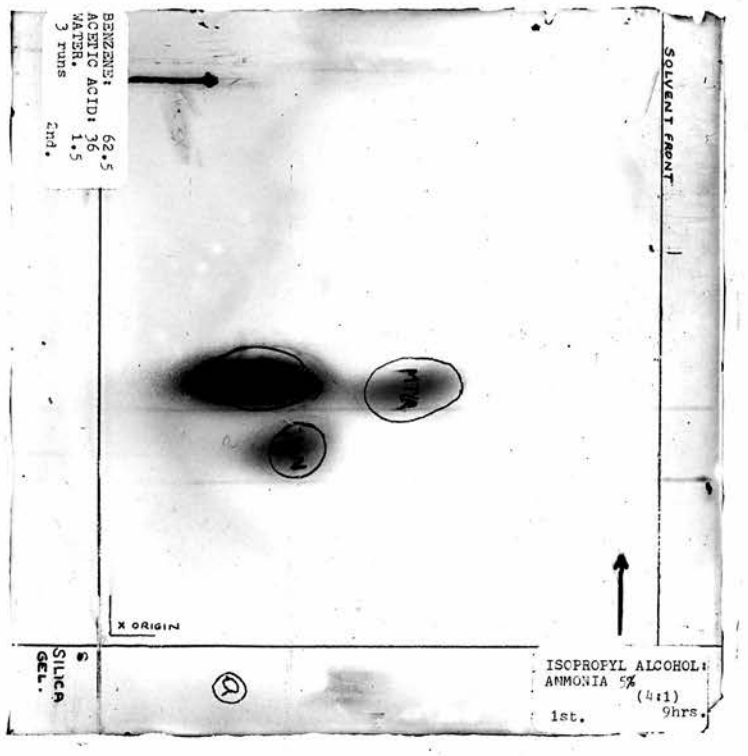
L-methionine and S-adenosylmethionine shown,
adrenaline and noradrenaline (not shown in
Figures) are separated from the catabolites.

Spots were visualized by spraying with diazotised
paranitroaniline, then ethanolic 1N NaOH.

O-METHYLATED AMINES

Two Dimensional Ascending Chromatography

(a)



1st System - Isopropyl alcohol:ammonia 5%
 (4:1 v/v) - single phase
 Developing time: 9 hours.

2nd System - Benzene:acetic acid (glacial):water
 (62.5:36:1.5 v/v) - single phase. 3 runs.

Thin layer precoated silica gel plate (20 x 20 cm.)

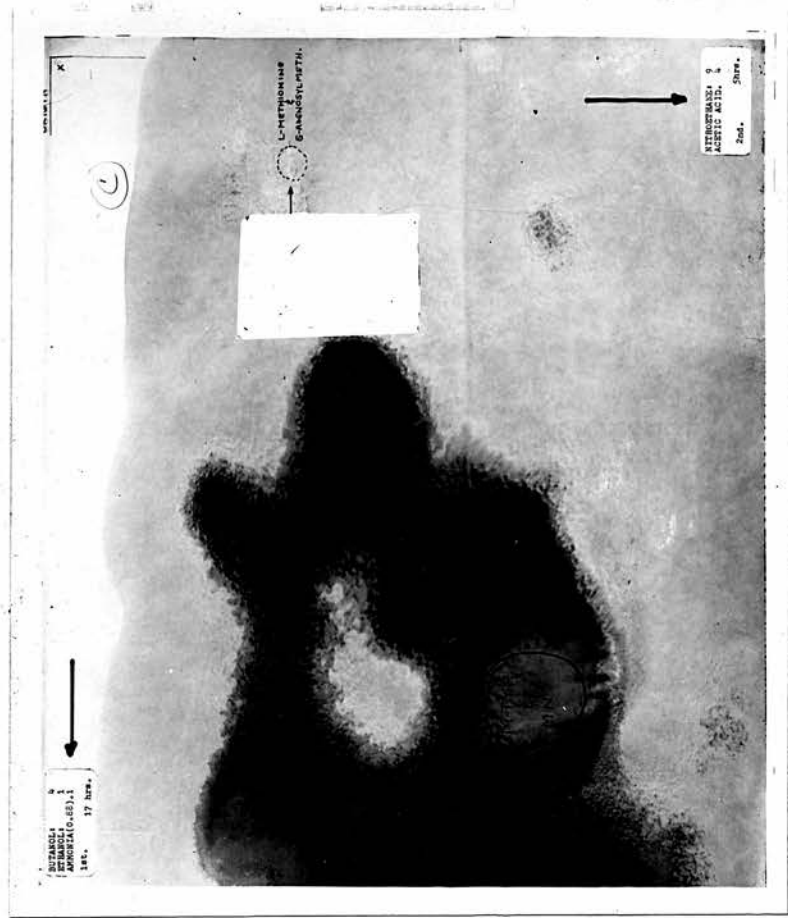
a,b,c:

L-methionine and S-adenosylmethionine (not shown in (a) but shown in (b) and (c)) plus noradrenaline and adrenaline (not shown in Figures) are separated from the catabolites.

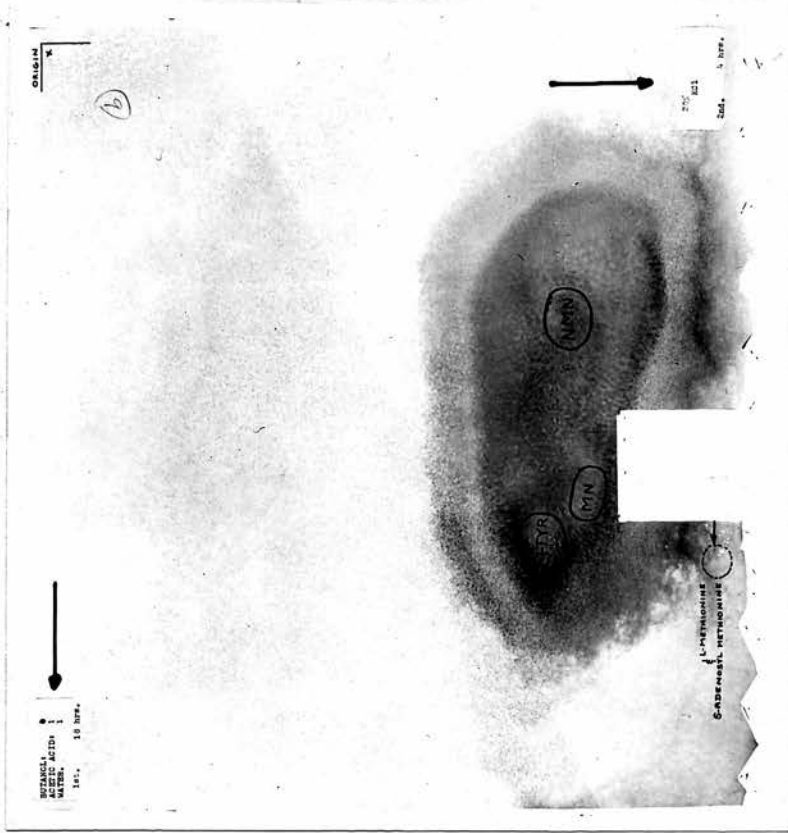
Spots were visualized by spraying with diazotised paranitroaniline, then ethanolic 1N NaOH.

Two Dimensional Descending Chromatography

(c)



(b)



Whatman No. 1 chromatography paper.

1st System → Butanol:acetic acid (glacial):water
(4:1:1 v/v) - single phase
Developing time: 18 hours.

2nd System → 20% KCL
Developing time: 4 hours.

1st System → Butanol:ethanol:ammonia (0.88)
(4:1:1 v/v) - single phase
Developing time: 17 hours.

2nd System → Nitroethane 70%:acetic acid (glacial)
(9:4 v/v) - single phase
Developing time: 5 hours.

APPENDIX VI

CALCULATED ANALYSIS OF RAT DIET

APPENDIX VI

CALCULATED ANALYSIS

Crude Oil	3.80%	Lysine	1.10%
Crude Protein	20.14%	Methionine	0.39%
Crude Fibre	2.54%	Cystine	0.36%
Digestible Oil	2.74%	Tryptophane	0.23%
Digestible Crude Protein	17.31%	Valine	0.96%
Digestible Fibre	1.16%	Leucine	1.30%
Digestible Carbohydrate	46.71%	Isoleucine	0.87%
Linoleic Acid	1.05%	Threonine	0.64%
		Phenylalanine	0.87%
		Tyrosine	0.61%
Metabolizable Energy		Histidine	0.49%
	2925 cal per kg.	Arginine	1.16%
Vitamin A	5355 i.u./kg.	Ca : P	1 : 1.1
Vitamin D ₃	1474 i.u./kg.	Calcium	7.6 gm/kg.
Vitamin E	100 mg/kg.	Phosphorus	8.1 gm/kg.
Vitamin B ₁	5.7 mg/kg.	Potassium	6.4 gm/kg.
Vitamin B ₂	8.0 mg/kg.	Sodium	4.8 gm/kg.
Vitamin B ₁₂	33.6 mcg/kg.	Chlorine	7.4 gm/kg.
Pantothenic Acid	18.1 mg/kg.	Magnesium	1.5 gm/kg.
Biotin	0.15 mg/kg.	Iron	214 mg/kg.
Pyridoxine	8.5 mg/kg.	Copper	30 mg/kg.
Inositol	0.22 gm/kg.	Manganese	46 mg/kg.
Choline	1.63 gm/kg.	Cobalt	1 mg/kg.
		Iodine	6 mg/kg.
		Zinc	6 mg/kg.

Diet (in pellets) containing 0.39% methionine fed to the control group of rats.

The experimental group were fed the same diet with added L-methionine as to constitute 6%.

PHYSICIANS AND NURSES' RATING SCALES

LORR SCALE: Was adapted from the "In-patient Multidimensional Psychiatric Scale" (Lorr et al, 1962). The scale consists of 18 items each item comprising 4 sub-items rated from 0-3 except for one item which is rated from 0-2. Thus the total maximal rating on the scale is 53.

The items cover affect, thought process, content and form, psychomotor behaviour, attention span, orientation and insight.

HENRY PHIPPS SCALE: (The Johns Hopkins Hospital - Psychiatric Clinic) -

The purpose of the scale is to obtain a graphic picture of patient's day to day behaviour during the trial. Recording is carried out daily by a nurse. Each 'day' consists of a 24 hours period beginning and ending at 7 a.m.

All items describe undesirable behavioural features. Each is scored 0-3 according to the nurses' global judgement of its severity/persistence during the 24 hours period.

The total maximum daily score is 108, however the maximal daily score recorded during the trial was 65.

HENRY PHIPPS NURSES RATING SCALE

Form 6151

ROYAL EDINBURGH HOSPITAL

Unit History No. _____

WARD _____

BEHAVIOR CHART

Psychiatric No. _____

NAME _____

Year..... 1959	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
Month..... JUN																															
Untidy.....																															
Exposed.....																															
Masturbating.....																															
Vulgar.....																															
Loosely creative.....																															
Distractible.....																															
Frankish.....																															
Impulsive.....																															
Destruative.....																															
Combative.....																															
Angry.....																															
Resistant.....																															
Agitated.....																															
Irritable.....																															
Restless.....																															
Talking.....																															
Calm.....																															
Industrious.....																															
Cheerful.....																															
Games.....																															
Reading.....																															
Corresponding.....																															
Lacking Initiative.....																															
Brooding.....																															
Preoccupied.....																															
Sad.....																															
Weeping.....																															
Picking, rubbing.....																															
Somatic Complaints.....																															
Apprehensive.....																															
Panicky.....																															
Confused.....																															
Indifferent.....																															
Eyes Closed.....																															
Motionless.....																															
Incontinent.....																															
Suicidal.....																															
Delusions.....																															
Hallucinations.....																															
Suspicious.....																															
Mute.....																															
Ritualistic.....																															
Peculiar Mannerisms.....																															
Sedative Medication.....																															
Packs.....																															
Baths—continuous.....																															
Baths—cleansing.....																															
Exercise..... 8																															
Occupation..... 7																															
Visitors..... 6																															
Menstruation..... 5																															
Defecations..... 4																															
Tubefed..... 3																															
Spoonfed..... 2																															
Eating..... 1																															
Sleeping, 1 sq. 1 hr. 0																															

EXCLUDED

EXCLUDED

PHYSICIAN'S RATING SCALE FOR CHRONIC SCHIZOPHRENIA

Adapted from the M.S.R.P.P. (Lorr Scale)

- P.1. How direct and relevant are his responses to questions or to the topic discussed?
- 0 Direct and relevant
 - 1 Somewhat rambling and tangential
 - 2 For the most part irrelevant
 - 3 Wholly irrelevant
- P.2 Does he assume or maintain peculiar, unnatural or bizarre postures?
- 0 None
 - 1 For short periods
 - 2 Throughout most of the interview
 - 3 Throughout the entire interview
- P.3 Are his thoughts consistent with his mood, or is there a discernible lack of harmony between them?
- 0 Consistent
 - 1 A little disharmonious
 - 2 Distinctly disharmonious
 - 3 Appear totally unrelated.
- P.4 Does he exhibit any repeated peculiar gestures, grimaces or mannerisms?
- 0 None
 - 1 Occasionally
 - 2 Fairly frequently.
 - 3 Throughout the interview
- N.5 Does he tend to suspect or to believe on slight evidence or without good reason that people and external forces are trying to or now do influence his behaviour and control his thinking?
- 0 No unjustified suspicion
 - 1 Inclined to suspect
 - 2 Believes others are trying to control him
 - 3 Believes he is influenced or controlled
- P.6 Are the elements of his speech logically consistent and connected by some idea of relationship, or do they tend to be inconsistent and disconnected? (Rate what is more representative during the interview.)
- 0 Coherent and consistent.
 - 1 Slightly incoherent and inconsistent
 - 2 Distinctly incoherent and inconsistent
 - 3 Conspicuously scattered, disconnected or incoherent.
- N.7 Does he bear little hostility or a high degree of ill will, resentment, bitterness or hate?
- 0 No hostility
 - 1 Slight hostility
 - 2 Moderate hostility
 - 3 Much hostility
- P.8 Does he have any insight into his hallucinations? (Score 0 for no evidence of hallucinations)
- 0 Full insight
 - 1 Possibly full insight
 - 2 Some insight
 - 3 No insight
- P.9 How frequently does he speak, mutter or mumble to himself, seemingly to carry on conversations with hallucinatory voices?
- 0 Not at all
 - 1 Occasionally
 - 2 Fairly frequently
 - 3 Throughout the interview

- P.10 Is there any evidence that the patient has auditory hallucinations?
0 None
1 Doubtful
2 Probable
3 Certain
- P.11 Does the patient ever glance up as if listening to auditory hallucinations?
0 Not at all
1 Doubtfully or occasionally
2 Fairly frequently
3 Throughout the interview.
- P.12 Does he repeat certain words or phrases in a meaningless, stereotyped or mechanical fashion?
0 Never
1 Occasionally
2 Fairly frequently
3 Almost constantly.
- P.13 Is his speech irregularly interrupted, halted or blocked for varying periods of time because of difficulty in finding words for his thoughts?
0 No speech blocks
1 A few interruptions
2 Many interruptions and conversations very difficult.
- N.14 Does he have an exaggeratedly high opinion of himself, or an unjustified belief or conviction of having unusual ability, knowledge, power, wealth or status?
0 No exaggerated high opinion of himself.
1 An exaggeratedly high opinion
2 Conviction of unusual power, wealth etc.
3 Conviction of grandiose or fantastic power, wealth etc.
- N.15 Does he tend to suspect or to believe on slight evidence or without good reason that some people are against him (persecuting, conspiring, cheating, depriving, punishing) in various ways?
0 No unjustified suspicions
1 Inclined to suspect.
2 Inclined to believe
3 Has firm conviction.
- N.16 Does he tend to suspect or to believe on slight evidence or without good reason, that some people talk about, refer to, or watch him?
0 No unjustified suspicions
1 Inclined to suspect
2 Inclined to believe
3 Has firm conviction.
- N.17 Is there evidence of false ideas or beliefs? If present, are these ideas or beliefs (a) sufficiently plausible as to be accepted by a normal person uninformed as to the facts, (b) implausible but not impossible, (c) impossible or bizarre (e.g.) mind controlled by radio waves, heart removed or dead)?
0 No evidence of false beliefs
1 Plausible to the uninformed
2 Implausible
3 Impossible or bizarre
- P.18 Does the patient's mood and emotional response show blunting?
0 Not at all
1 Slight blunting
2 Severe blunting
3 Complete apathy.

~~H. Waller~~

464 b

HANDBOOK
of
PSYCHIATRIC
RATING SCALES
(1950-1964)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
Public Health Service

INTRODUCTION: Some Principles of Rating Scale Design and Use

The use of ratings of some sort to record or to classify judgments or observations is ancient. There are many hundreds of words in our language that are evaluative terms, and many of them have quantitative implications with respect to their referents (e.g., "good-better-best"). A *rating scale* consists of a defined continuum or dimension (hot-cold, active-inactive) along which judgments are placed. The scale may be a set of ordered categories with numbers attached; it may be a series of adjectives, phrases, or statements arranged in a sequence corresponding to an increasing or decreasing amount, severity, or other quantitative characteristic of the phenomenon being observed; or it may be a line on a page with its end-points labeled with two numbers (e.g., 0-100 or 0-10) upon which the rater makes his estimate of the degree or amount of the observed phenomenon with respect to some standard. The "standard" in psychiatric and psychological research is usually a class of persons, such as chronic schizophrenics, normal adolescents, or some other reference group, and the task of the rater is to locate graphically or numerically the individual under consideration with respect to the appropriate reference group. For example, if a psychiatric patient is judged to be about average in his reference group in a certain trait (say, "overt hostility") and is to be rated on a scale having numerical indices ranging from 0 to 10, then a rating of 5 would be appropriate.

There are other instruments, perhaps improperly called scales, requiring that the judge make discrete responses to a number of items. A list of adjectives that the rater checks "yes" or "no," depending upon whether the item is or is not descriptive of the subject or patient, is an example of this kind of device. For many of these, "scores" are computed by counting the number of responses in one or several categories, perhaps attaching different weights to certain items, depending upon their differential significance for the purpose at hand. If

these scores are conceived as defining a quantitative dimension corresponding to a trait or characteristic under study, then we may consider such a score to represent a point on a scale, provided certain other basic requirements are met.

For many assessment instruments numerical indices from several closely related scales or scales believed to form a useful or meaningful constellation or syndrome may be added into a single score. A descriptive name may be given to such a score, which may be called a "scale." Sometimes an entire instrument comprising a number of such composite scales may be called "a scale." This can lead to some confusion, particularly when investigators use rating devices constructed by themselves and do not give adequate descriptions of their instruments in published reports.

In the scale reports in this *Handbook* we have usually followed the author's own terminology but have attempted in the scale descriptions to clarify the usage of words such as "item," "scale," "score," "cluster," etc., where necessary. In this introductory discussion an individual statement, question, or adjective to which a rater is required to make a response will be called an *item*. A numerical index derived from responses to a set or group of items will be called a *score* or *scale score*.

In recent years much attention has been given to the "theory" of scales from the mathematical point of view. Principles and methods have been developed in attempts to make ratings and other psychological measures comparable in some of their characteristics to physical measurements (e.g., that a difference of 5 points represents the "same" difference in magnitude anywhere within the scale's range in the same way that differences in pounds or inches are equivalent). There are both philosophical and technical issues here. Elaborate procedures have been devised for the construction and scoring of scales to achieve or approximate some of these aims, but they have had little influence upon makers

or users of psychiatric rating scales, and there is little or no evidence that the usefulness of these scales would be significantly increased by such refinements.

A Historical Note

Sir Francis Galton was one of the first investigators to apply systematic rating methods in behavioral science. Late in the 19th century he devised an eight-category scale and asked his subjects to evaluate the "vividness" of their visual images.

Soon scales of various types were being used in psychological laboratories to quantify judgments of sensory and perceptual responses. The application of ratings in the educational and personnel (industrial and military) areas thrived during and after World War I, and many of the principles of scale construction and the analysis and use of ratings were developed in these situations and during this period. By and large, these principles apply to psychiatric rating scales today.

The use of rating scales and similar devices for psychiatric purposes developed more slowly, although a pioneer example was the Phipps Psychiatric Clinic Behavior Chart, constructed and used at Johns Hopkins about half a century ago. At least several such instruments were devised during the 1920's and 1930's but their use appears to have been primarily local, i.e., for a specific research purpose or within a single hospital.

During and following World War II the rapidly growing concern over the problems of mental health and illness, the stimulation of research by the military services, the Veterans Administration, the National Institute of Mental Health, state governments and private foundations, and the increasing research sophistication of professional persons in the mental health field, have led to the development of a number of assessment devices that can yield information suitable for research as well as other purposes. The recent introduction of tranquilizers and other psychologically active drugs and the encouragement of controlled drug tests by the Psychopharmacology Service Center of NIMH have further stimulated scale use and scale development.

Advantages of Quantitative Rating Scales

Interview Guide.—Many clinicians have reported that a schedule of items covering a variety of observable symptoms and relevant questions concerning patients' attitudes and feelings helps to

assure a more thorough and complete psychiatric interview. There is less likelihood that possibly significant phenomena may be omitted or overlooked. This is valuable, whether or not the information so derived is later reduced to numerical form for other purposes.

Communication.—In contrast to narrative descriptions, numerical or graphic ratings are more likely to be interpreted within the same semantic frame of reference. Clinicians with different backgrounds and holding different theoretical views find it valuable to have on record information gathered in a systematic and common format. This can be of considerable importance when patients are reassigned to other wards within a hospital or to other physicians.

Record of treatment progress.—A scale or set of scales appropriate to the disorder under treatment ("valid" in one of the meanings described in another section of this survey) can be an extremely useful device for recording behavioral or other trends indicative of changes during the course of treatment. Numerical or graphic ratings of presence-absence or severity of symptoms can serve a purpose similar to a "fever-chart" in recording treatment results.

Research.—Efficient and productive research requires objective data, data that can be manipulated statistically, data that have sufficient dependability ("reliability"), and data obtained in such a way that other researchers can repeat the study in an attempt to confirm (or challenge) the original findings. The most sensitive of modern research designs demands information in numerical form, derived from the same instrument or instruments, and gathered in a uniform or reproducible manner. Well-constructed rating scales, as other psychometric devices, when properly used, meet most of the requirements of the most elaborate and sophisticated of today's experimental and statistical procedures.

Format.—The major varieties of scale format are illustrated by the descriptions and sample items given in this *Handbook*. Usually the rater's task is to check one of several words, phrases, or statements making up a given item. In some scales the rater is free to make a mark at any point along a line indicating the degree or magnitude of the characteristic being reported, and the position of the mark may then be translated into a number for

scoring purposes. Generally the typography and the physical arrangement of items on the page reflect attempts to facilitate the rater's job and the subsequent handling and scoring. In some scales the item format is deliberately varied to minimize the likelihood that a rater will fall into a response pattern, such as making most of his ratings in the same direction or in a limited part of the scale.

Another format that has seen increasing use in recent years is the card-sort scale. Statements or adjectives are printed on individual cards and the rater's task is to sort them into several piles or stacks (4, 5, or 7 are frequently used) ranging from "most descriptive" to "least descriptive." Essentially, this does not differ from the usual numerical or graphic format (in fact, some scales have been produced and used in both forms). It may have several advantages in certain rating situations: (1) The "novelty" may appeal to some raters. (2) The rater is not likely to skip items inadvertently. (3) A judgment is less likely to be influenced by the immediately preceding ratings. (4) If the items are printed on pre-punched I.B.M. cards, the piles may be stacked in order and taken directly to machines for scoring or other statistical treatment. On the other hand, in some projects problems of handling, storage, and manual scoring may make this method less efficient. Difficulties will also arise if the results, including the individual rater responses, are wanted as a document of record.

Psychometric Principles and Characteristics

This is an introduction to some key concepts of reliability and validity. The discussion is necessarily simplified, and many readers familiar with basic psychometric theory may want to omit it.

The Correlation Coefficient

A tool frequently used in evaluating reliability and validity of ratings is the *correlation coefficient*. This is a numerical index expressing the extent of agreement between two sets of data (e.g., ratings on scale A and scale B for a group of patients, or ratings on the same scale by Judge 1 and Judge 2). There are several ways of deriving and computing such a coefficient, depending upon the characteristics of the data and the purpose of the investigator. All have in common the fact that the range of values is from -1.0 through zero to a maximum of $+1.0$, with -1.0 reflecting perfect or complete disagreement; zero, chance agreement or no relation-

ship; and $+1.0$, perfect agreement. With a correlation of $+1.0$ between scales A and B, knowing the value of one scale for an individual permits perfect prediction of the other. (This would imply, of course, that the two scales supply the same information and that one—either one—is unnecessary.) A graphic presentation of a perfect correlation (on a "bivariate diagram" or a "scatterplot") might look as follows:

HIGH 5					2	
4				11		
Scale B Scores 3			19			
2		5				
1	6					
LOW 0	1					
	0	1	2	3	4	5
	LOW	Scale A Scores			HIGH	

In this diagram the numbers in the lower-left to upper-right cells represent numbers of individuals in the rated samples. Note that a subject's position on one of the six-category scales corresponds precisely to his position on the other. A perfectly *negative* correlation would yield a configuration with a reversed appearance—the frequencies would be distributed from upper left to lower right. (Actually, however, this could have precisely the same interpretation as the positive case and can be transformed into it by merely reversing the direction of scoring, e.g., by assigning high numbers to that end of the scale previously identified by low numbers.) A correlation of zero means that the two variables have merely a chance relationship with each other, and that knowledge of one for an individual is of no value at all in predicting the other. Such a case might look like this:

HIGH 5			2	2		
4		4	6	6	4	
Scale B Scores 3	2	6	10	10	6	2
2	2	6	10	10	6	2
1		4	6	6	4	
LOW 0			2	2		
	0	1	2	3	4	5
	LOW	Scale A Scores			HIGH	

Note that here, within each row or column, the frequencies distribute themselves about the central tendency or the mean of the entire group.

Actually, *perfectly* zero correlations (and perfectly positive ones) are almost never found in research. But the statisticians have devised methods for assessing the "significance" of correlation coefficients to determine the degree of confidence with which we may conclude that a relationship exists or that a given coefficient is "significantly different from zero."

These methods, with the results usually expressed in percentages (most commonly .05 and .01), are interpreted to mean that if the "true" correlation were in fact zero, chance fluctuations in the sample of data at hand would give rise to a coefficient as large as that found in no more than 1 in 20 (or 1 in 100) repetitions of the study using similar samples.

Factor Analysis

In the discussions of some of the rating scales in this report there are references to "factor analysis," "factors," or "factor loadings." Factor analysis is a fairly complex set of arithmetical operations that begins with a table displaying the correlations of each variable under consideration (scale or item) with every other one. Then further computations are performed leading essentially to a *reduction* in the number of variables or dimensions that "account for" or "explain" the observed relationships among the original set of data. Thus, for example, after a factor analysis of 100 rating scale items, it may be concluded that six factors are present, which would indicate that the 100 items are yielding information about six different aspects or modes of behavior. A "factor loading" is a numerical index (with a maximum value of 1.00) reflecting the degree of association that a particular item has with the factor concerned.

For rating scales, factor analysis has been used for two major purposes: (1) to identify clusters or families of items that "hang together" and can be scored as a scale or sub-scale, thereby reducing the amount of information an interpreter or user must consider, and (2) to organize a mass of data in such a way that an investigator can more easily understand it or formulate or test hypotheses concerning the meaning or significance of the behavioral dimensions that his scales are measuring.

The reader should be warned that differences in purpose, differences in theoretical points of view,

and differences in mathematical techniques are such that detailed results of one factor analysis may not necessarily be comparable to those of another, even though the same variables are involved.

Reliability

The term "reliability" refers to several different but related concepts in psychometric theory. Basic to all of them is the notion of "measurement error," which recognizes the fact that test scores, ratings, and other quantitative observations are subject to fluctuations from a hypothetical "true" value that itself cannot be determined with assurance of perfect accuracy but is "estimated" by the observed score or rating. The smaller this error, the more "reliable" are the data. Independent observations that do not agree with each other are presumed to contain some "error," although it may not be possible to discover whether the error is in one set of data or both, or which set is the more dependable. On the other hand, observations that agree highly or even perfectly may contain error factors if they have been subject to similar distortions; as, for example, when ratings are obtained by the use of a poorly-constructed instrument or are made by different observers sharing the same systematic biases.

Indices of Reliability

One obvious index of reliability is agreement among two or more raters. There are two standard methods of assessing such agreement: (1) by determining the proportion of judgments assigned to the same categories by both members of a pair of raters or (2) by computing the correlation between their numerical ratings. These two indices of agreement have different properties and do not convey the same information. Consider these extreme examples of what might happen with 50 patients being rated on a 5-point scale by two judges:

		2			
4					
3		3			
Judge B 2			40		
1				3	
0					2
	0	1	2	3	4
	Judge A				

Here the percentage of agreement is substantial—80% (since 40 of the 50 patients were rated “2” by both judges), but the correlation coefficient is —1.00.

On the other hand, consider this second case:

				10	
4					
3			15		
Judge B 2		15			
1	10				
0					
	0	1	2	3	4
	Judge A				

In this case the percentage of agreement is exactly zero, since no patient was assigned to the same category by both judges. The correlation coefficient however, is 1.00!

In the first example the two raters were obviously in complete disagreement concerning the trait or symptom they were observing. As “measuring instruments” they were performing in precisely opposite directions, and their 80% agreement consisted merely in recognizing the neutral or mid-point of the scale they were using.

In the second case, the two judges appear to be in complete agreement on the meaning of the scale and their observations seem to be equally sensitive (within the limitations of the scale’s format), but they obviously have different frames of reference or different “zero-points,” since one judge’s ratings are consistently one point higher than the other’s.

Both kinds of information are valuable. A high inter-rater agreement would ideally mean that one rater could be chosen at random and his ratings could be used interchangeably with those of another. For this to be the case, we would want *both* a high correlation *and* a high degree of agreement on category placement.

If the ratings of two judges indicate significant disagreement in either of these two senses, one may conclude that (1) the trait or characteristic is elusive, ill-defined, or not ratable, (2) the wording of the item or the response alternatives is unclear or misleading, or (3) the raters (or at least one of them) are not sufficiently perceptive or not properly trained for the task. It should be noted, however, that two raters may be in perfect agreement as a result of sharing the same biases or the same

distorted understanding of their task or of the phenomena they are observing.

There are other methods of estimating reliability widely used in psychometric work. One is the *test-retest* (or *rate-rerate*) coefficient, the correlation between sets of ratings made at different times (say, with the lapse of a day or a month). It is thus a measure of the stability of the trait or characteristic over a period of time. This index has certain advantages when used with tests of cognitive abilities or of certain “basic” personality variables that would not be expected to change significantly with the passage of time. But with mental patients, especially with an active treatment program, one expects changes in behavior, and the longer the time interval, the greater the likelihood of significant change. For this reason, the *rate-rerate* correlation is likely to be a distorted index of reliability. If the time interval is shortened in an effort to overcome this difficulty, another problem arises in that raters in a conscious or unconscious effort to be “consistent” may be influenced on the second occasion by memory of their first ratings.

Reliability may also be estimated by so-called “internal consistency” indices. These are estimates of how well items in a test or a scale “hang together” with respect to the characteristic or trait they presume to measure. Consistency, homogeneity, equivalence, and “purity” are terms that have been used to describe it. A “split-half” coefficient may be computed by dividing the items within a scale into two sets (randomly or by taking odd-versus even-numbered items) to form the two halves and adjusting the correlation obtained so that it is appropriate to the entire set rather than to half. This method, used with some rating scales, is not suitable for many, because a given scale frequently includes only a small number of items.

Other consistency measures are variations of the “Kuder-Richardson” coefficient, often abbreviated as K-R. These coefficients, like the standard or “product-moment” coefficients, can range in value up to +1.00, but are not exactly comparable numerically. The most widely used of this class were originally developed for ability tests scored 1-0; right-wrong, or yes-no. More recently, they have been generalized for test items and scale items having a numerical range extending beyond a dichotomous or two-category scoring system. High K-R coefficients result when items which are responded to in a positive direction by one subject or

rater receive positive responses from most other subjects or raters; and similarly for negative responses.

Another reliability index sometimes used is the *intra*class coefficient. This measure is valuable in situations where, for example, one may have two or more ratings for each patient but they are not made by the same set of raters. The first patient may have been rated by rater A and rater B, the second by raters A and C, the third by raters D and E, etc. It is clearly impossible to compute for the entire sample of subjects a standard correlation coefficient expressing agreement between any *pair* of raters. The *intra*class coefficient is a statistic that in effect ignores rater identity and considers all raters interchangeable. Since differences in rater standards and rater skills are not taken account of, the *intra*class coefficient can be expected to be numerically smaller than an ordinary correlation computed from ratings made by the same team of raters for all patients. Values as high as .80 have been reported for some scales, indicating agreement quite satisfactory for most research purposes.

One problem of evaluating reliability indices for check lists and ratings is the fact that in many collections of items a considerable amount of agreement is "built into" such instruments as a consequence of the semantic structure of our language. One can go to a thesaurus or a dictionary of synonyms and assemble lists of adjectives and be confident that judges would respond to the lists similarly. For example, a widely used scale includes in one score the terms "tired" and "fatigued." A study of depression used a symptom inventory containing the items "decreased appetite" and "diminished appetite." To the extent that such pairs (or larger groups) of items evoke similar responses from raters, consistency coefficients are thereby larger, but with no corresponding increase in the amount of information contained in the scale scores.

The question of how large the reliability coefficient for a given population should be to justify the use of scale results has no precise answer, since it depends upon how the ratings will be used, the kind of coefficient, the number of cases, etc. For clinical use, i.e., in making decisions concerning diagnosis or treatment of individuals, many clinicians would want reliabilities as high as .80 or .90. For research purposes, to test hypotheses concerning group differences, treatment comparisons, etc., lower reliabilities may be acceptable, particularly

where reasonably large numbers of cases are involved. If treatment differences are sufficiently great, scales with reliabilities as low as .50 or even .40 may differentiate the groups and enable the investigator to reach a conclusion. On the other hand, if the discrimination is not statistically significant, one is left uncertain as to whether more reliable measures would have led to more confident conclusions.

In addition to the standard techniques there may be indirect evidence that may assure an investigator that the instrument he is using may be sufficiently reliable for his purpose without, however, providing a numerical index. If it is found that a scale significantly differentiates two or more treatment groups or predicts an outcome criterion (is "valid" in one of the senses discussed below) then one may assume that it is reliable. The reasoning here is that if the ratings were reflecting merely "chance" or "error," they could not possibly make such discriminations or predictions; and this is the basis for one of the elementary principles of psychometric theory: that a score cannot be valid for any purpose unless it has reliability.

Validity

Validity in psychometric theory has to do with whether and to what degree a test or rating device accomplishes the purpose its user intends. Depending upon the purpose, several kinds of validity may be distinguished:

Predictive validity.—This concept of validity is of most concern to the typical user of rating scales or to one who wishes to choose among the large number available a scale suitable for his particular study or experiment. Predictive validity is oriented toward a criterion or outcome in which an investigator is interested. The purpose in one type of study may be to compare the effectiveness of two or more methods of treatment, and the question is whether a scale can detect significant differences among the treatments, hence among the groups of patients, thereby leading the investigator to a decision or recommendation.* Another form of predictive validity is concerned with the use of a score (or some combination of scores) to "predict" a later outcome variable for an individual. A familiar example is the use of intelligence test scores as an aid in the selection of college students, or performance

* This has been called "discriminative validity" by some writers.

tests in the selection of industrial workers. In the psychiatric setting such validity information would be useful in deciding which patients should be admitted to which type of treatment. Other things being equal, a patient could be assigned to that treatment for which his predicted outcome is most favorable. The usual index of this second form of predictive validity is the correlation coefficient as described above.

Three additional kinds of validity are distinguished, but they are ordinarily of less concern to the user of psychometric instruments than is predictive validity. They are, however, of considerable importance in the construction, development, and evaluation of certain types of scales, since they deal largely with properties of the scales themselves. Following are brief definitions of these:

Concurrent Validity.—This concept deals with the comparability of two sets of measures (e.g., score and criterion) determined at essentially the same time. (There is nothing to be predicted, since the criterion data are already known.) The extent to which scale scores can differentiate currently diagnosed groups of psychiatric patients is an example of this type of validity.

Content Validity.—This refers to a test or scale made up of items *randomly* drawn from a population or pool of items assembled because they represent some area of interest to the investigator. The term "content" derives from the fact that the content of the random sample is an acceptable reflection of the content of the parent pool and is hence "by definition" valid.

Construct Validity.—If a score is intended to be a measure of some hypothetical characteristic which itself cannot be directly observed or measured, the attempt to verify, at least provisionally, that the test or scale yields results not inconsistent with the investigator's hypothesis, involves the concept of construct validity. "If my hypothesis concerning the dynamics underlying such-and-such personality dimension is tenable, then my rating scale should have such-and-such characteristics (form of score distribution, correlation with other measures, differences in scores between Groups A and B, etc.)." "If I am correct in my interpretation of a certain Rorschach score, then this score from my rating scales should correlate highly with it." These are examples of construct validity.

Concluding Remarks on Reliability and Validity

It is important to keep in mind that there is no such thing as *the* reliability or *the* validity of a rating scale. A reliability coefficient is not an intrinsic property of the scale for which it may have been reported. Factors affecting reliability and validity include the skill and motivation of the rater, the rater-scale combination or "team" (including the rater's attitude toward or "rapport with" the scale used), the range or variation in the trait or symptom under observation, and the conditions under which the ratings were obtained—to name several of the more important ones. In the scale summaries that follow, the reader should not assume that he will obtain results comparable to those reported for a given scale *even when the experimental or treatment situation appears to be similar or identical*.

Norms

Early in the history of intelligence testing and educational testing the producers of tests were aware of the need for "yardsticks" for evaluating an individual's standing with respect to other members of a group, to compare the achievement of a school class with that of other classes, etc. Large and well designed samples of the population or populations with which the test is intended to be used are needed for dependable "norms" (from the Latin *norma*, a rule). Separate norms for such characteristics as age, sex, school grade, or combinations of these, have been compiled for many tests. It should be noted that, although published norms for standard tests have been extremely useful, few have escaped criticism on technical grounds, such as size and appropriateness of samples, etc.

For several of the scales discussed in this report, norms are reported by the authors. Most of these are difficult to evaluate because of insufficient information concerning the samples used or because they are largely local, i.e., obtained from one or a very few hospitals or other sources. With differences in admission practices, diagnostic policies, social and racial composition, etc., from hospital to hospital, the investigator is advised not to take these norms too seriously. Perhaps the most dependable norms are those assembled by the Veterans Administration for scales developed in their programs (especially when used with patients comparable in age, sex, etc., to the VA hospitalized populations).

Data from the NIMH-PSC collaborative studies will be valuable as normative information, since the hospitals participating approach the goal of a stratified sample of hospitals.

Research involving the use of rating scales can be carried out successfully with no reference to

norms at all, provided the scales have acceptable reliability and validity. Many investigators, however, will wish to establish their own local norms, as a matter of interest or as a "quality control" device to help assure that rating standards are reasonably stable over time.

The Inpatient Multidimensional Psychiatric Scale

LORR, M., MCNAIR, D. M., KLETT, C. J. and LASKY, J. J. Evidence of ten psychotic syndromes. *J. consult. Psychol.*, 1962, 2, 26, 185-189.

LORR, M., KLETT, C. J., MCNAIR, D. M. and LASKY, J. J. *Inpatient Multidimensional Psychiatric Scale*. Palo Alto: Consulting Psychologists Press, 1963.

General description.—The Inpatient Multidimensional Psychiatric Scale consists of 75 brief questions designed to measure 10 psychotic syndromes. The clinicians' ratings are based on observations of patient behavior and verbal reports of beliefs, attitudes and feelings as obtained in an interview of 30 to 45 minutes. Completion of the form should require 10 to 12 additional minutes.

Type of patient.—Functional psychotics or severe psychoneurotics who can be interviewed. Those inaccessible are better described with a ward rating form.

Type of rater.—Trained interviewers and observers experienced in interviewing psychiatric patients and familiar with psychiatric symptomology. It is suggested that the rater explain the purpose of the interview, be nondirective, and leave direct questioning or probing to the end. It is recommended that there be an interviewer and a silent observer.

Basis for rating.—The focus of the interview should be on discernible behavior and on patient self-reports of current feelings, attitudes and beliefs. The emphasis is on current behavior or experience. Social history, previous interview notes and reports of ward observations are to be disregarded.

Source of items.—The scale is a complete revision of the MSRPP (Multidimensional Scale for Rating Psychiatric Patients, Lorr, 1953), a rating

schedule designed to record descriptions of psychotic behavior and symptoms. Many of the items were rewritten and certain other changes were made. The "Unratable" category of the MSRPP was eliminated, bipolar scales were converted to unipolar form, and two sets of adverbs were used to characterize the intensity and the frequency of behaviors reported on the five- and nine-point scales.

The first experimental form consisted of 42 nine-point scales and 48 dichotomous items. Ratings were collected on four to ten patients in each of 47 hospitals by teams consisting of an interviewer and a silent observer independently rating each patient. Patients were selected as being representative of one of the following patterns by a professional person other than the interviewer:

"Anxiously depressed, markedly delusional, hostile-belligerent, well-integrated paranoid, apathetic and unmotivated, markedly hallucinated, disorganized in thought processes, retarded and depressed, and manic excited."

The sample consisted of 296 patients. Correlations among the 77 variables selected for analysis were computed and 10 syndromes or clusters were found.

Revision of the experimental form was based on the initial analysis, a study of inter-rater reliabilities, and other considerations. The final form was then employed in obtaining a normative sample of 566 cases on which the score norms (centiles) are based.

Application of a further analysis based on hypothesized groupings of variables yielded the same 10 syndromes as the first analysis. "Each syndrome is regarded as a unitary pattern of response present to a greater or lesser degree in all patients. Further, it is assumed that the more severe the syndrome, the more probable that deviant behaviors will be manifested." They are each defined by a group of

items measuring in common a "unitary reaction tendency." The 10 scores are largely independent. Intercorrelations range from $-.37$ to $.56$, with a median of $.16$.

Type of items.—Statements in non-technical language are arranged without regard for their relationship to a particular scale. In this report the syndromes are listed and described, with examples of each.

A. EXCITEMENT (EXC). Characterized by an excess and acceleration of the individual's speech and motor activities and also by lack of restraint in the expression of emotions and feelings. Mood level and self-esteem are usually high.

COMPARED TO THE NORMAL PERSON TO WHAT DEGREE DOES HE

1. Express or exhibit feelings and emotions openly, impulsively, or without apparent restraint or control?

Cues: Shows temper outbursts; weeps or wrings hands in loud complaint; jokes or talks boisterously; gestures excitedly.

9. Manifest speech that is hurried, accelerated or pushed?

Cues: Pressure of speech.

B. HOSTILE BELLIGERENCE (HOS). A complaining, griping, attitude, manifest hostility; and an inclination to express resentment towards and to feel suspicious of others' intentions are evident in this syndrome.

5. Verbally express feelings of hostility, ill will, or dislike of others?

Cues: Makes hostile comments regarding others such as attendants, other patients, his family, or persons in authority. Reports conflict on the ward.

11. Express a feeling or attitude of contempt, disdain, or scorn towards other people as unworthy or beneath him?

Cues: Derogatory or snide comments about others; sarcasm or ridicule of others; condescending.

C. PARANOID PROJECTION (PAR). The syndrome is defined by beliefs that attribute a hostile and controlling intent to the world around the patient.

TO WHAT EXTENT DOES HE APPEAR PREOCCUPIED WITH OR DISTRESSED BY

44. Delusional beliefs or convictions?

59. Does he believe that some people talk about, refer to, or watch him?

D. GRANDIOSE EXPANSIVENESS (GRN). The syndrome appears to represent a stage beyond paranoid projections. The individual characterized by this syndrome has found an explanation for his persecution. He is really an important personage with a divine mission and unusual powers.

COMPARED TO THE NORMAL PERSON TO WHAT DEGREE DOES HE

15. Exhibit in demeanor and/or in verbalizations an attitude of self-importance, superiority, or conceit?

Cues: Speech is pompous or stilted; boasts of his accomplishments; demands and expects special privileges.

HOW OFTEN DID HE

54. Hear voices that praised, extolled, or spoke to him about divine missions?

E. PERCEPTUAL DISTORTION (PCP). Hallucinations that threaten, accuse, and demand define this syndrome. The underlying mechanism is one of distortion of sensory and perceptual stimuli. Voices say "bad" things about the patient.

TO WHAT EXTENT DOES HE APPEAR PREOCCUPIED WITH OR DISTRESSED BY

45. Hallucinatory voices?

HOW OFTEN DID HE

53. Hear voices that accuse, blamed, or said "bad" things about him, e.g., that he is a spy, homosexual, murderer?

F. ANXIOUS INTROPUNITIVENESS (INP). Three elements appear to characterize this syndrome: anxiety, turning against the self, and lowered mood level. The reaction is one in which the individual experiences guilt and remorse and holds himself to blame for real or imagined faults.

COMPARED TO THE NORMAL PERSON TO WHAT DEGREE DOES HE

14. Tend to blame, criticize, condemn, or otherwise hold himself responsible for past or present, real or fancied, thought or actions?

Cues: Blames self for failure, difficulties, and frustrations in family relations, work, or finances.

21. Report or admit being uneasy or anxious in anticipation of specific future difficulties or problems?

Cues: Worried about his symptoms, his family, or his finances.

G. RETARDATION AND APATHY (RTD). The syndrome is defined by a slowing down and reduction of ideation, speech, and motor behavior. At the extreme there is apathy and disinterest.

COMPARED TO THE NORMAL PERSON TO WHAT DEGREE DOES HE. . . .

1. Manifest speech that is slowed, deliberate, or labored?

8. Exhibit indifference or apathy towards such matters as his treatment, his release from the hospital, or plans for the future?

Cues: Content to stay. Willing to "leave it to the doctor." Sees no need for treatment. Seems to have no goals and no expectations.

H. DISORIENTATION (DIS). The syndrome measures varying degrees of disorientation. As represented here, it probably is not due to brain injury but is indicative of autism or intense self-directed attention.

DOES HE KNOW. . . .

70. That he is in a hospital?

71. In what state the hospital is located or the nearest large city?

I. MOTOR DISTURBANCES (MTR). Rigid, bizarre postures; grimacing, and repetitive movements are the principal behaviors defining this syndrome.

COMPARED TO THE NORMAL PERSON TO WHAT DEGREE DOES HE. . . .

6. Exhibit postures that are peculiar, unnatural, rigid, or bizarre?

Cues: Head twisted to one side, or arm and hand held oddly. Judge the degree of peculiarity of the posture.

10. Manifest overt signs of tension?

Cues: Moves or shifts restlessly; body musculature appears taut, strained or tense; fingers clothing, scratches, drums or fiddles with objects, face or neck muscles twitch; exhibits startle reactions, palms sweaty.

J. CONCEPTUAL DISORGANIZATION (CNP). Disturbances in the stream of thought evidenced in irrelevant answers and incoherent or rambling speech characterize this syndrome. These suggest disorganization of thinking processes.

COMPARED TO THE NORMAL PERSON TO WHAT DEGREE DOES HE. . . .

2. Give answers that are irrelevant or unrelated in any immediately conceivable way to the question asked or topic discussed?

Cues: Do not rate here wandering or rambling conversation which veers away from the topic at issue. Also, do not rate the coherence of the answer.

3. Give answers that are grammatically disconnected, incoherent, or scattered, i.e., not sensible or not understandable?

Cues: Judge the grammatical structure of his speech, not the content, which may or may not be bizarre.

Scoring.—The dichotomous items are given values of 0 and 1; the five-point scales, values from 0 to 4; the nine-point scales values from 0 to 8. Differences in scale range are adjusted when the scales are combined into a syndrome score by assigning weights of 8, 2 and 1 for the two-point, five-point, and nine-point scales, respectively, to equalize approximately the contribution of each item to the syndrome score. The syndrome scores for one rater are obtained as follows:

A. For scores other than DIS and INP find the sum of weighted ratings on the scales defining the syndrome.

B. The score for DIS is the sum of the ratings defining DIS multiplied by -1 plus a constant of 6.

C. The INP score is the sum of the weighted ratings defining INP plus a constant of 8 which is needed to avoid negative values introduced by one scale with a negative weight.

The syndrome scores when ratings are available from two raters are computed separately, then added together. The materials used in scoring are the *score sheet* and the *centile norms*. The scale value of each rating is recorded on the score sheet and then the weighted ratings are added. For DIS and INP the necessary constant is added. The centile rank corresponding to the weighted score for each syndrome is provided by tables that show how a given patient stands with respect to the patients in the norm population. For example, the centile rank of 70 on the Excited syndrome means that the patient is as much or more excited than 70 per cent of the norm population.

The morbidity scores.—In addition to these syndrome measures, three "morbidity scores" were derived from an analysis of the correlations among the 10 syndrome scores.

The first measure is bipolar and defines an Excitement versus Retardation pattern. The score is the sum of EXC and HOS minus RTD.

The second pattern is one of tendency toward distortion of thinking and perception. To score, add the syndrome scores of the patient on PAR, PCP and GRN.

The third pattern is one of schizophrenic disorganization. To obtain the raw score, add syndrome scores on MTR, DIS, CNP and RTD.

Reliability.—Degree of inter-rater agreement was determined by obtaining two independent ratings on 508 patients in the norm sample and computing the intraclass coefficients for each scale. These reliabilities ranged from .82 for MTR to .91 for GRN. The authors point out that somewhat lower reliabilities are to be expected when only one rater is used.

Reliabilities of individual items were computed and found to range between .61 and .92.

Validity.—In one study reported in the Manual all scores except INP, RTD, and DIS were found to discriminate significantly between open ward patients and closed ward patients in the expected directions, i.e., those on closed wards showing a greater degree of disturbance as indicated by higher scores for seven syndromes.

If the IMPS scores are to be useful in diagnosis, one would expect them to have some relation with the usual psychiatric classifications. For 190 patients assigned to six standard diagnostic categories, those groups designated paranoid schizophrenia, depression, and manic-excitement are well described by appropriate scores. The remaining groups, schizophrenia (mixed), hebephrenia, and catatonia are not clearly delineated.

In one of the projects of the VA Cooperative Studies of Chemotherapy in Psychiatry (1960) the

IMPS, along with other measures, was used in an attempt to identify differential changes in schizophrenic patients treated with several drugs. The major findings have greater relevance to the question of the efficacy of the treatments than to that of the validity of the IMPS. It is interesting to note, however, that several scores, HOS, PAR, PCP, and CNP, were significantly related to patients' scores on a scale of attitudes toward medication. The more paranoid patients have less faith that medication will help them. Similar results were obtained with the Psychotic Reaction Profile (PRP) for these same patients.

The four variables that make up the Schizophrenic Disorganization morbidity score, RTD, DIS, MTR, and CNP, were found to be significantly related to duration of hospitalization, suggesting a usefulness for this score in predicting hospital stay.

The Abbreviated IMPS.—The authors have selected from the complete set a group of 20 items, two for each syndrome, which can be used for a rapid assessment if necessary. Although scores on the brief form agree closely with those on the complete form (the median correlation is about .88), the authors warn that they are necessarily less reliable and contain less information. Norms for the syndromes and the morbidity scores are provided.

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Psychotic Reaction Profile

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General description.—A behavior rating scale to measure ward behavior factors by means of 85 statements rated True or Not True by a nurse or attendant on the basis of a few days observation of ambulatory functional psychotic patients. The scales are labeled Withdrawal, Thinking Disorganization, Paranoid Belligerence and Agitated Depression. Centile norms by sex and age are available for each scale and also norms for 16 unique patterns.

Type of patient.—Functional psychotics, hospitalized, ambulatory.

Type of rater.—Ward personnel: nurse, nursing assistant or hospital aide. The rater should be trained in the use of the scale.

Basis for rating.—The patient is to be rated solely on observations of hospital behavior for the three days preceding the rating.

Source of items.—Four hundred behavior statements were constructed by scrutinizing published inventories, rating scales, reports of factor studies and psychiatric texts. A group of clinical psychologists familiar with psychotic behavior allocated the statements to one of nine areas of behavior disturbance, all of which have been repeatedly identified in multiple-factor studies of interview and ward behavior. The hypothesized primary constructs were Resistiveness and Motor Disturbances (catatonic-like behavior), Withdrawal versus So-

ciability, Perceptual Distortions, Conceptual Disorganization, Melancholy Agitation, Paranoid Projection, Hostile Belligerence and Lack of Interest in Self or Others. In addition an interpersonal parameter of Dominance versus Submissiveness was postulated. A final list of 172 items distributed among the nine subscales was retained.

Data for approximately 1000 functional psychotic cases in 47 hospitals were analyzed and four essentially homogeneous and independent groups of items were identified and labeled Withdrawal, Thinking Disorganization, Paranoid Belligerence, and Agitated Depression. Intercorrelations among the four are low (ranging from .05 to .40), indicating a satisfactory degree of independence.

Type of item.—The Withdrawal Scale consists of 38 statements, appears to be bipolar and is defined by three discernible elements: activity level, manifest interest in the environment, and interpersonal responsiveness. One pole of the scale defines a withdrawal reaction consisting of a lack of interest or apathy, a failure to respond to others and a reduced activity level. The opposite pole describes an excessive busyness, overactivity, and a high level of interpersonal reactivity. Examples of items are: 1. Usually stays by himself; 5. Ignores the activities around him; 13. Seems concerned about what others think of him; 24. Nearly always chatting with someone.

The *Thinking Disorganization Scale* of 18 items defines a broad parameter, probably central to schizophrenia. Disorientation, irrelevant and incoherent speech, hallucinations and peculiar movements are characteristic of this pattern. Examples of items are: 4. Occasionally talks to himself; 8. Answers sensibly when talked to; 12. Sees and hears things that are not there; 20. Spends a lot of time talking to himself.

The *Paranoid Belligerence Scale* of 24 items describes patients who are hostile, irritable, resistive, bossy and paranoid. To some clinicians this pattern

would represent a hostile acting out. Examples of items are: 2. Tells the other patients what to do; 6. Sometimes does the opposite of what he is asked to do; 10. Acts as though the ward attendants are against him; 25. Is likely to hit someone for no apparent reason.

The *Agitated Depression Scale* consists of five items which appear to suggest anxiety and obvious depressive elements. The items are: 3. Shows real sadness; 7. Seems to be unhappy; 11. Usually looks tired and all worn out; 15. Seems scared all the time.

Scoring.—The rater is asked to mark statements as True or Not True. Items are arranged in random order so that scale groupings are not apparent. Centile norms by sex and age are available for each scale. Norms are also available for 16 unique patterns.

Reliability.—Internal consistency reliability estimates were computed. All coefficients except that for Agitated Depression are at least .90. To ascertain inter-rater reliabilities, data were obtained from a state hospital on a group of 54 male and 52 female psychotics, each rated by two attendants. The correlations indicate that all scales except the Agitated Depression scale have a satisfactory reliability. The Withdrawal scale is the most reliable of the scales developed. The authors consider that the reliabilities compare favorably with those reported for typical achievement and aptitude tests.

Validity.—One aspect of validity was investigated by determining whether the scales could differentiate between open and closed ward patients, since the former are likely to be less severely ill. Two independent samples of 55 male open and 110 male closed ward patients were compared on each of the four subscales. Thinking Disorganization scores were significantly higher for closed ward patients in both samples. Withdrawal scores were significantly higher for the closed ward patients at the .10 level in sample A and at the .025 level in sample B. Agitated Depression separates the two groups in expected directions at the .10 level in both samples. Paranoid Belligerence fails to differentiate the two groups in either sample. To test whether unique patterns on the four scales could differentiate open from closed ward patients, each scale was dichotomized and sixteen unique patterns constructed, with each patient allocated to one pattern. The utility of the patterns was tested on a sample of 110 open ward patients and 220 closed ward

patients. Correct identification was made of 80 per cent of the closed ward patients, but open ward patients could not be identified on a better than chance basis.

When the initial keying procedure was applied separately to the data based on the descriptions of male and female patients, they failed to reveal any differentiating items. The order of inclusion of items in a cluster was not always the same, but the final keys were essentially identical for both sexes. The norm data also tended to support the conclusion that the psychotic reaction patterns do not differ greatly for men and women. When each of the four scale score distributions was examined for sex differences, only Paranoid Belligerence differed significantly. It is possible that this type of behavior is tolerated more by ward personnel in women and is likely suppressed or penalized in men.

Factor analysis.—An attempt to confirm that PRP measured certain ward behavior factors was made by Lorr and O'Connor (1962), using 500 male psychotic patients. The ten postulated dimensions were Manic Excitement, Withdrawal with Psychomotor Retardation, Paranoid Projection, Perceptual Distortion (Hallucinations), Conceptual Disorganization (Incoherent and Disordered Speech), Resistiveness, Depressive Agitation, Motor Disturbances (Manneristic Postures), and Hostile Belligerence. A personality variable of Dominance versus Submission was also introduced as potentially useful in discriminating between patients. Analysis revealed the following factors:

Factor TD—Thinking Disorganization (Ward)

Spends a lot of time talking to himself	.75
Talks to himself about imaginary or real faults	.65
Sees and hears things that are not there	.59
Occasionally talks to himself	.58
Smiles a lot to himself without sensible reason	.58
Repeats words and phrases in a meaningless way	.56

This factor includes diverse elements previously considered to be distinct, such as motor disturbances, hallucinations and thinking disturbances. The underlying disorder seems to be broadly indicative of schizophrenic disorganization and disintegration as conceived by Kraepelin and Bleuler.

Factor PR—Paranoid Projection (Ward)

Blames hospital for lack of attention and care	.65
Complains about the food and care he receives	.61
Acts as though the ward attendants are against him	.59
Acts as though the hospital is persecuting him	.52
Often irritable, grouchy or complaining	.47

The Paranoid Projection (Ward) factor describes a tendency in a patient to feel that people are

against him, to attribute hostile motives to others, and to grouse or complain in dissatisfaction.

Factor HB—Hostile Belligerence (Ward)

Often swears and uses obscene language	.53
Sometimes threatens to assault others	.50
Often shouts and yells	.48
Loses temper when dealing with other patients	.48
Becomes noisy and hilarious at times	.41

The factor labeled Hostile Belligerence has been identified a number of times in other studies and in most cases this syndrome is positively associated with morbid suspicion as exemplified by the Paranoid Projection factor.

Factor R—Resistiveness (Ward)

Resists suggestions and requests from aides	.41
Sometimes does the opposite of what he is asked to do	.38
Has to be pushed to follow routine	.38

Resistiveness represents a tendency, high in catatonics and paranoid patients, to resist helping, working or complying with instructions, as well as a tendency to do the opposite of what is asked.

Factor D—Dominance (Ward)

Bosses the other patients	.74
Tells the other patients what to do	.65
Acts superior to other patients	.47
Upsets other patients by the way he talks to them	.38

The factor of Dominance has not been found in ward or interview data previously. The authors believe it is more likely a personality characteristic than a psychotic reaction.

Factor O—Overactivity (Ward)

Is always doing something	.84
Seems always busy with plans and projects	.66
Starts conversations with aides to become better acquainted	.38
Will do anything for recreation that comes up	.37

The factor tentatively labeled Overactivity (Ward) is characterized by overactivity and busyness.

Factor AD—Agitated Depression (Ward)

Shows real sadness	.57
Usually worried and nervous	.52
Seems to be unhappy	.44
Seems scared all the time	.43
Usually looks tired and all worn out	.36

The Agitated Depression (Ward) factor implies a reaction of fear, anxiety and psychomotor retardation. It is strongly suggestive of depression with agitation.

Factor W—Withdrawal (Ward)

Never says more than three or four words at a time	.54
Laughs or smiles at funny comments or events	.48
Doesn't take part in back and forth conversation	.45

Usually stays by himself	.45
Never volunteers information about himself	.39
Shows occasional interest in news and current events	—34
Never asks for anything; waits for things to be given him	.36
Likes to go for exercise	—33
Ignores the activities around him	.32
Shows no response to entertainment	.32
Shows real friendliness towards at least one other patient	—31
Interested in nothing	.31

The Withdrawal factor (Ward) is well represented in the inventory. The parameter is defined by statements indicative of taciturnity or failure to respond in conversation, avoidance of interactions with others, and lack of interest in the surrounding environment.

At the present time the Psychotic Reaction Profile is being used in a number of studies, notably those in the Veterans Administration cooperative series. An example is a study of the relations of attitude toward medication to treatment outcomes in psychotherapy (Gorham and Sherman, 1961) by administering a short scale measuring attitude toward medication before and after 20 weeks of treatment of 369 chronic schizophrenic patients assigned to five drug groups. Also used were the PRP and IMPS (Inpatient Multidimensional Psychiatric Scale), with the PRP keyed to yield seven measures, and the IMPS, 10 measures. The PRP measures were Thinking Disorganization, Withdrawal, Paranoid Belligerence, Agitated Depression, Resistiveness, Dominance and Activity Level.

The hypothesis that the attitude of patients toward their treatment is related to treatment response as measured by the 17 criteria was tested for 80 per cent of the patients studied. It was found that *changes in symptom areas were not related to patient belief* in the efficacy of medication. The relationship between *attitude toward medication* and *pretreatment symptom measures* were found to be statistically significant for six of the criteria; IMPS—Paranoid Projection, five drug groups were analyzed separately, several relationships were found, suggesting that symptom relief was related to positive attitude toward medication.

RELATED REFERENCES

- GORHAM, D. R. and SHERMAN, L. J. The relation of attitude toward medication to treatment outcomes in chemotherapy. *Amer. J. Psychiat.*, 1961, 117, (9), 830-832.
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