DEMETHYLATION, METHYLATION & SCHIZOPHRENIA:

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PREFACE

The work to be described investigates the hypothesis that 'faulty transmethylation' occurs in schizophrenia. This hypothesis has been used to explain a variety of biochemical and psychopharmacological observations in that condition. An alternative hypothesis of faulty demethylation in schizophrenia is also subjected to investigation. The original impetus for the experimental work was a claim by Friedhoff and Van Winkle (1962), that they had discovered an amine, 3:4 dimethoxyphenylethylamine (D.M.P.E.) in urine from schizophrenics but not in urine from control subjects, a claim which many would now regard as discredited.

The experimental section is divided into three parts.

1. Preliminary studies of the demethylation hypothesis were carried out in vitro. The demethylating enzyme system in human liver was shown to be:-

   a) Dependent on the cofactors T.P.N. and nicotinamide.

   b) More readily able to demethylate D.M.P.E. in the 4-O position. (which demethylation can be regarded as a detoxification process).

   c) Capable of demethylating the drug METHACETIN. Evidence suggesting that D.M.P.E. and Methacetin are demethylated by the same enzyme is presented.

An initial intention to compare the ability of liver from

non-schizophrenics with that of liver from schizophrenics to demethylate a suitable substrate was found to be impracticable, because of difficulty in obtaining liver from schizophrenics.

2. The use of a pharmacogenetic approach (pharmacogenetics can be defined as a study of genetically determined variations in animal species which are revealed by the effects of drugs) suggested itself as a way of circumventing the need to obtain liver from schizophrenics. Using such an approach, the 4-O demethylation of methacetin by two control groups and two schizophrenic groups has been compared in vivo.

The first control group comprised university technicians and students; the first schizophrenic group comprised acute schizophrenics. Of the first schizophrenic group, one subject showed abnormal results which various further investigations did not satisfactorily explain.

The second control group was of older non-schizophrenic mental hospital patients and the second schizophrenic group one of older chronic schizophrenics. Results gave no support to the demethylation hypothesis.

3. The faulty transmethylation hypothesis was subjected to investigation in vivo, using pharmacogenetic technique.

The substance chosen for the methylation study was 3:4 dihydroxy benzoic acid (protocatechuic acid, PCA) and measurements were made of the urinary metabolites of this compound.

Methylation of PCA occurs predominantly in the 3 position (yielding vanillic acid, VA), but methylation in the 4 position also occurs (yielding isovanillic acid, IVA). Since 4-O-methylated compounds generally have more psychopharmacological activity than the corresponding 3-O-methylated compounds, the ratio VA/IVA was considered to be particularly worthy of study.

In order to have some assessment of the 'rate of methylation,' the ratio between the total (i.e. unconjugated plus conjugated) VA excretion and the excretion of PCA was derived.

Two further investigations were carried out:-

a) into urinary creatinine content,

b) into the production of 3:4 dimethoxybenzoic acid as a metabolite of PCA (a limited study, with negative findings).

A statistical analysis is presented, showing that-

a)'rate of methylation' and VA/IVA ratios did not differ significantly between control subjects and schizophrenic subjects.

b) The urinary excretion of unconjugated vanillic acid was pH dependent.

c) The 'rate of methylation' index was related significantly to body weight in females, both in normals and schizophrenics, but not in males (both sexes receiving an identical dosage schedule). It is suggested on this and other evidence that vanillic acid may penetrate body fat.

d) There was a direct relationship between 'rate of methylation' and IVA/VA ratios : both may reflect the level of availability of methyl groups needed for transmethylation to occur.

e) There was high correlation between the amounts of VA and IVA excreted .

There was more scatter in creatinine values in schizophrenics than in control subjects. This may relate in part to their clinical state, agitated and frightened patients having high values, apathetic and inert patients low values.

These results are discussed in the context of previous work and their implications considered.

The advantages and disadvantages of a pharmacogenetic method of approach to a problem such as that of schizophrenia (where ethical difficulties, including that of 'valid consent,' limit research) are considered.

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LITERATURE SURVEY

Introduction.

A great deal has now been published on biological aspects of schizophrenia, but in recent years there has been more concentration on specific hypotheses than had previously been the case. Hitherto, as new techniques for the study of psychological, physiological or biochemical function had become available, they were inevitably applied to the unsolved problem of schizophrenia. Inevitably, because for many years schizophrenics formed a (literally) captive population on which a large study could quickly be mounted when a new technique arrived; furthermore any unsolved problem may always yield to investigation from a previously untried approach, however empirical this may be. In such a situation spuriously 'positive' results must just as inevitably appear, either by chance or for reasons some of which can be understood as being the results of the incarceration of patients within a mental hospital. Among such reasons one might instance the effects of a custodial atmosphere, the effects of lack of exercise, the effects of drugs, and the effects of the gut bacteria and of diet.

It would be unreasonable to discount this earlier work as being invalid or irrelevant to the essential biochemical lesion if such

there be. For example, the impaired ability of the chronic schizophrenic to conjugate glycine with hippuric acid has been found by a number of workers (see Richter, 1957) and a satisfactory explanation of their observations has yet to be found.

Recently there has been a focussing by biological psychiatrists, psycho-pharmacologists and biochemists from a number of centres on a specific hypothesis; moreover, this specific hypothesis is not empirically derived but relates to the resemblance that exists between schizophrenia and mescaline intoxication. This hypothesis which has become known as the 'faulty transmethylation' hypothesis originated in a paper produced by Osmond, Harley-Mason and Smythies (1952) under the title "Schizophrenia : a new approach." After comparing the detailed symptomatology of acute schizophrenia with that of intoxication with the methylated compound mescaline and finding much in common between the two conditions, the authors considered the possibility that aberrant O-methylation of noradrenaline (which is normally converted to adrenaline by N-methylation) might produce either:

4-methoxy 3-hydroxy phenylethanolamine or

3,4-dimethoxy phenylethanolamine.

Osmond, Harley-Mason and Smythies went on to discuss the mechanism of such changes with special reference to the adrenal glands and made the suggestion that "the process of methylation becomes

disturbed and highly toxic substances are produced."

Since that time a considerable amount of work (which will be dealt with below) has been devoted to this "faulty transmethylation hypothesis" and fifteen years later Seymour Kety (1967) wrote-

"That hypothesis has by no means been proved nor is it likely to be proved very easily. It remains, however, a remarkably parsimonious explanation for a mass of otherwise disparate information and for that reason appears to be worth entertaining and testing."

A detailed consideration of evidence relating to the "faulty transmethylation" hypothesis of schizophrenia.

Evidence relating to this hypothesis derives from a number of separate lines of investigation.

a) Methylated compounds as hallucinogens.

It has already been mentioned that the methylated compound mescaline (3,4,5 trimethoxyphenylethylamine) induces an abnormal state in normal subjects not without resemblance to the clinical situation in acute schizophrenia. Compounds having similar properties to mescaline are some of the N-dimethyl and N-diethyl derivatives of tryptamine. These include dimethyltryptamine, psilocyn, psilocybin and lysergic acid diethylamide (L.S.D.). In the latter there is a psilocybin-like part and a nicotinamide-like part. These are hallucinogens capable of producing hallucinations

in a setting of clear consciousness (or "true" hallucinogens, to use a descriptive label sometimes applied to drugs possessing this property). In this they differ from a number of other drugs, e.g. those belonging to the atropine group (the "deliriants") in which hallucinations are accompanied by clouding of consciousness.

Hollister (1962) criticised the claim that the hallucinogenic drugs produce a schizophrenia-like state although his own descriptions to some extent belie his objections, since he noted many similarities between the drugs states and acute schizophrenia. He commented that patients with a drug induced state showed delusions rarely; that their hallucinations often occurred in the non-auditory sensory modalities; that they did not show catatonic features; and that (unlike the schizophrenics) they showed concern at their failure to communicate. Hollister's experimental technique used "blind" ratings of taped interviews of schizophrenics and of normal subjects experiencing a drug induced state. His conclusions that the raters could distinguish the two conditions are open to question (as he himself admitted) since clues of tense, accent and diction interfered with complete rater "blindness."

That points of difference exist between the clinical findings in schizophrenia and those in drug induced states is, nevertheless, generally conceded even by those who appear most impressed by their similarities (Delay, 1962). Some have drawn attention to the

likelihood that differences may be expected between a time limited drug experience in an experimental setting and a continuing schizophrenic illness; others to the fact that the drug subject, unlike the schizophrenic, knows that changes are likely to take place and that their experiences are, to this extent, not comparable.

Studies of the clinical states induced by the hallucinogens would seem to afford some support to the hypothesis that there may be an endogenous psychotogen in schizophrenia. It must be admitted, however, that no drug or combination of drugs has yet been found which mimic schizophrenia precisely. However, even if such a hypothesis were proved and a psychotogen isolated and administered, other factors (such as those mentioned above) might prevent perfect mimicry.

It is of interest that although mescaline has a very different chemical constitution from psilocybin and L.S.D., there is evidence that the drugs all act by a similar mechanism : not only do they produce the same type of symptoms but they show a pronounced cross-tolerance (Isbell et al., 1961; Wolbach et al., 1962). The placing of the methylated compounds mescaline, L.S.D. and psilocybin in a special group (of "true hallucinogens") on symptomatological grounds is therefore consistent with pharmacological observations.

Smythies (1968) in a discussion of this topic, has suggested

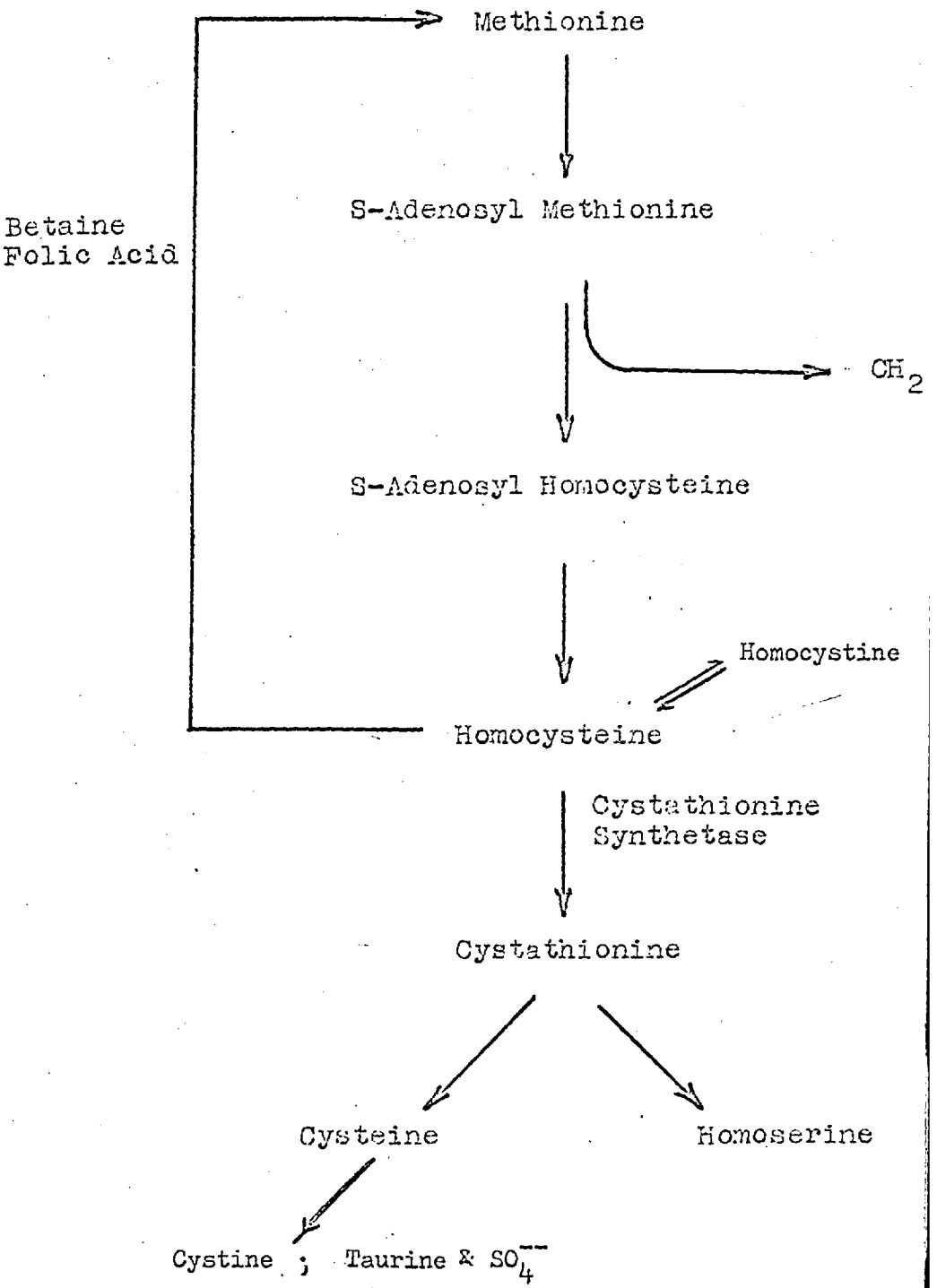
that "it is surely significant that the hallucinogens are all either O-methyl or N-methyl derivatives of the cerebral neurohumors, noradrenaline, dopamine and 5 hydroxytryptamine."

b) The effects of feeding methyl donors to schizophrenics.

A number of reports have been published (Pollin et al., 1961; Brune and Himwich, 1962; Alexander et al., 1963; Berlet et al., 1965a; Haydu et al., 1965; Park et al., 1965 and Kakimoto et al., 1967) on the effects of feeding methyl donors to schizophrenics. Usually the methyl donor has been methionine (occasionally betaine) and (except in the work of Haydu et al., 1965), a mono-amine oxidase inhibitor (M.A.O.I.) has been given in order than any amines formed would not be broken down so readily by monoamine oxidase.

A wide range of symptoms and signs has been noted to occur as a result of this medication; some authors have claimed the symptoms to be largely 'toxic,' and mention such changes as "delirium, visual hallucinations, ataxia, speech disturbance, increased salivation and hyperhidrosis" (Kakimoto et al., 1967), flushing and tremors (Alexander et al., 1963), orthostatic hypotension and cardiac arrhythmia (Berlet et al., 1965a) and impaired memory span (Park et al., 1965). These symptoms have tended to occur when higher doses of either methionine or M.A.O.I. (or both) have been given; schizophrenics on lower dosage have frequently shown no toxic symptoms but manifested a clinical state in which existing symptoms of

Figure 1 The Metabolic Pathway from Methionine to Cysteine



schizophrenia have been intensified or new symptoms have developed, e.g. the appearance of word salad, and an 'upsurge of hallucinatory activity' (Pollin et al., 1961), increased delusions and hallucinations (Park et al., 1965) and increased withdrawal and increased disorganisation of thought (Berlet et al., 1965a).

A number of objections can be raised to the work so far carried out in this field. First, there has been a paucity of studies on non-schizophrenic subjects, although Alexander et al (1963) reported studies on two such subjects (both psychoneurotics) and stated that behavioural changes similar to those in the schizophrenic group were produced. However, this report mentioned no symptoms which one would regard as specifically schizophrenic. Secondly, some workers have administered l-methionine; some dl-methionine while others have not specified the type of methionine used, thus hindering a direction comparison between the different studies. It must be admitted, however, that perusal of the various reports does not suggest that the type of methionine used makes any consistent difference to the results obtained. Thirdly, in several series the period during which tranquilliser therapy was withdrawn prior to the onset of methionine/M.A.O.I. administration has been undesirably short. Nussbaum (1964) has pointed out that withdrawal of tranquilliser therapy may lead to a "pseudostimulation effect" characterised by increase of agitation and psychotic ideation;

Pollin et al. (1961) produced increased anxiety with an increasing flood of associations with methionine and M.A.O.I. indicating that the misinterpretation of drug withdrawal effects is more than a theoretical pitfall.

Finally, too few studies such as that of Pollin et al. (1961) have been carried out with amino acids other than methionine. An exception is the study by Spaide et al. (1967), who fed cysteine and M.A.O.I. to schizophrenics and who claimed exacerbation to have resulted. This at once complicates the issue, for cysteine is not a methyl donor although it does supply a thiol group which probably has an important part to play at the receptor site for the methylating enzyme catechol O-methyl transferase (Axelrod, 1966).

It has been suggested by Spaide et al. (1967) that "the rise in creatinine excretion (i.e. during cysteine administration) may indicate that muscle protein was catabolised thus releasing a variety of aminoacids including methionine." Another possibility suggested by these authors is that, as methionine is normally metabolised to cysteine, cysteine administration may lead to a "build up in methionine." Rose and Wixom (1955) have shown in this connection that cystine has a sparing effect on the human requirement for methionine. It will be seen from Fig. 1 that the normal pathway from methionine to cystine has cystathionine as an intermediary. Cystathionine formation (from serine and homocysteine) is catalysed

by the enzyme cystathionine synthetase; its breakdown (to homoserine and cysteine) is catalysed by the enzyme cystathionase. Kato et al. (1964a) have shown that cystine (which can be produced from cysteine) has an inhibitory effect on cystathionine synthetase activity, in vivo, in rats; and that both cysteine and cystine competitively inhibit cystathionase (Kato et al., 1966). It would appear likely, therefore, that cysteine administration in man may have a considerable effect on the methionine to cysteine pathway.

Sprince (1967) has suggested a further alternative mode of action for methionine. As a result of feeding excess methionine (or cysteine) to rats he found evidence of an inhibition of the tryptophan → kyneurenine → N_1-methylnicotinamide pathway, and evidence of an increase in the tryptophan → tryptamine pathway. He suggested that this may favour the formation of N-methyl derivatives of tryptamine, some of which (as has already been mentioned) are psychotomimetic.

It is important to note that the administration of the combinations methionine/M.A.O.I. or cysteine/M.A.O.I. increases the urinary excretion of non-methylated amines (and presumably increases tissue levels also) more than does M.A.O.I. alone. This holds for the non-methylated indoleamine tryptamine both with the methionine/M.A.O.I. combination (Sprince et al., 1963) and with the cysteine/M.A.O.I. combination (Spaide et al., 1967). Kakimoto and co-workers

(1967) studied the elevation in urinary amine levels (using a methionine/M.A.O.I. combination) of four amines, one non-methylated (noradrenaline), one N-methylated (adrenaline), one O-methylated (normetanephrine) and one both O- and N-methylated (metanephrine). A methionine/M.A.O.I. combination administered to eight long-stay hospital female schizophrenics led to the excretion of larger amounts of all four amines than did the M.A.O.I. alone, again showing that the effect of methionine is not limited to an increase in the production of methylated amines.

It may be noted that there is good evidence that M.A.O.I. therapy alone may exacerbate (particularly during the acute phase) or even precipitate schizophrenia. Price and Hopkinson (1968) in a review of this topic drew attention to the fact that, as far as chronic patients are concerned, M.A.O.Is. (used as "psychic energizers") seem to do little harm. Reports of exacerbation occurring with a methionine/MA.O.I. combination given to chronic patients therefore suggest that methionine makes a significant contribution to such exacerbations. That the contribution of methionine is important receives further support from the work of Pollin et al. (1961) who used a variety of amino acids but only induced symptoms of exacerbation with methionine (and M.A.O.I.) and from the experimental designs used by Bruno and Himwich (1962) and Park et al. (1965) which included a period in which M.A.O.I.

was administered alone, this therapy failing to exacerbate schizophrenia in patients in whom the methionine/M.A.O.I. combination later succeeded.

To summarise : there is evidence that a methionine/M.A.O.I. combination causes a greater rise in urinary amines (and presumably in tissue amines) than does M.A.O.I. alone; this effect is not limited to methylated amines for it occurs also with non-methylated amines (e.g. noradrenaline : Kakimoto et al., 1967 and tryptamine, Sprince et al., 1963). There is evidence that methionine (plus M.A.O.I.) can exacerbate the specific psychopathology of schizophrenia; this might be due to the excessive formation of a methylated amine or to the excessive formation of a non-methylated amine or to some other property of M.A.O.Is.;(which can inhibit a considerable number of enzymes in addition to monoamine oxidase, Gey et al., 1963); the precise mode of action of methionine in this situation is obscure. As far as the faulty transmethylation hypothesis is concerned, the evidence of the M.A.O.I./amino acid feeding studies is not inconsistent with the hypothesis, but affords it little decisive support.

c) The effects of reducing dietary methionine.

Berlet et al. (1965b) have examined the effect of reducing both dietary methionine and, simultaneously, dietary tryptophan on schizophrenic symptomatology. Their patients were nine male

chronic schizophrenics whowere maintained on the usually quoted 'minimum daily requirement' of L-methionine (1.10G.) for four weeks, followed by an eight week period on about a third of this amount (0.35 - 0.40G. per day). During the whole of this twelve week period dietary tryptophan was cut to about the minimum daily requirement (0.25 - 0.35 G/day). No consistent clinical changes were produced by this regime : worsening that took place in three patients was not clearly related in time to any particular dietary regime. However, there was biochemical evidence that a significant reduction of methyl groups was not induced by the restricted diet. Thus, although the urinary levels of N_1-methyl nicotinamide (which is formed by the methylation of nicotinamide) were reduced, these were brought back nearly to normal levels by nicotinamide administration. On this piece of information one can base a criticism of Berlet's work. Normally nicotinamide can be derived from dietary L-tryptophan. Nicotinamide is a methyl acceptor, and any situation in which levels of this compound fall below normal will tend to cancel out the effect of dietary methyl donor restriction by blocking one route for elimination of methyl groups from the body. It would, therefore, have been better to allow adequate dietary tryptophan whilst methionine reduction was in force and to study tryptophan withdrawal as a separate experiment.

d) The metabolism of C^{14} labelled methionine in schizophrenics.

Israelstam et al. (1967, 1970) reported a difference between normals and schizophrenics in the metabolism of methyl-C^{14}-labelled methionine. After they administered this substance, they collected expired air and from this derived the ratio of $C^{14}O_2$ to unlabelled CO_2. In normal subjects they found that this ratio reached a peak in about 20 minutes thereafter falling slowly; in acutely disturbed schizophrenics the peak was absent, a plateau which lasted for several hours occurring. The data were considered consistent with a) a rate-limiting enzymatically based step delaying the conversion of $C^{14}H_3$ (the labelled methyl group) to $C^{14}O_2$, or b) a larger methionine or transmethylating pool in schizophrenics than in normals.

Israelstam et al. (1970) reported an abnormal result for the test (although the abnormality was of a different type) in patients with depression, suggesting that the explanation for the findings relating to schizophrenics may not lie specifically in disordered transmethylation.

It is noteworthy that the total recovery of ^{14}C as $^{14}CO_2$ was of low order (in normals and schizophrenics about 2% in a 4-hour period) and also that studies were not extended to other biologically important compounds which may be predicted to have been labelled (e.g. choline, betaine, creatine, creatinine, N^1-methyl nicotinamide and the methylated catecholamines, to name but a few).

The studies are therefore open to the criticism that they give an incomplete picture of the situation.

e) The effects of methionine sulfoximide on schizophrenic and non-schizophrenic subjects.

Krakoff (1961) administered methionine sulphoximine to seven patients (non-schizophrenic) with advanced malignancy and showed that it was capable of inducing a psychosis characterised by hallucinations, disorientation and agitation. Symptoms such as those mentioned occurred within 3 - 5 days of starting oral methionine sulphoximine in a daily dose of 200 - 400 mgm. In one patient co-administration of a methionine : methionine sulphoximine mixture in a ratio of 19:1 produced no abnormality but administration of methionine sulphoximine in lower doses produced the psychotic symptoms, suggesting that methionine afforded some protection against the action of methionine sulphoximine.

Heath et al. (1966) fed methionine sulphoximine to nine prisoner volunteers and noted "autism, thought deprivation, disturbances of affect, blocking and association defects." Catatonic symptoms, delusion and auditory hallucinations also occurred in some of these subjects. Heath found, however, that methionine sulphoximine had quite a different effect on ten chronic schizophrenic subjects (who had been off medication for at least six months). They actually improved on the drug, as shown by their scores on several different rating scales, with most marked improvement in the effective sphere. This was exemplified by a

mute catatonic patient who "began to converse pleasantly with ward personnel and even danced the Charleston on one occasion." Only in memory testing did both schizophrenics and non-schizophrenics show the same response to the drug : deterioration. Memory, however, is characteristically unaffected by a schizophrenic illness. Heath listed the ways in which he thought methionine sulphoximine might have been acting-

(i) as an antimetabolite to methionine (inhibiting the incorporation of methionine into protein).

(ii) as an inhibitor of enzyme systems involved in the synthesis of glutamine and the production of bound acetyl choline, and

(iii) possibly by interfering with brain transmethylation.

Of these modes of action, the third can only be described as highly speculative. Heath's findings might support the transmethylation hypothesis if methionine sulphoximine could be shown to act by depriving an over-active methylating system of the methyl donor methionine, in the schizophrenic. Such an explanation, however, would not readily explain the mimicry of schizophrenia observed with methionine sulphoximine in the normal subject. Further clinical experimentation carried out on an entirely double-blind basis (which Heath's work was not) coupled with in vitro or in vivo studies on the effects, if any, of methionine sulphoximine on

transmethylation (either in brain or elsewhere) are clearly required. It would be also of interest to see whether methionine sulphoximine is metabolised similarly by control subjects and schizophrenics.

f) The effects of penicillamine in schizophrenia.

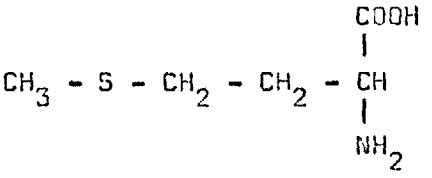
It seems unlikely, on the available evidence, that the effects in schizophrenia of the substance penicillamine afford support for the "faulty transmethylation" hypothesis.

Nicolson et al. (1966) took ten male chronic schizophrenics (who were maintained on long-standing phenothiazine regimes throughout) and fed five of these patients on 1,200 mgm. of D-penicillamine daily for six periods of six days, the other five receiving placebo. A low copper diet (approximately 1 mgm./day) was administered throughout to all ten patients.

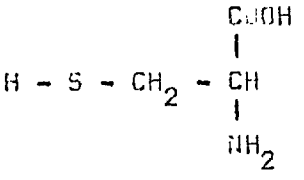
They found striking clinical improvement in the five patients treated with penicillamine, but no change in clinical state occurred in four patients receiving placebo, while a 'slight change' was noted in the fifth. Their complex hypothesis has been summarised as follows (Nicolson et al, 1966) :-

"Owing to a congenital defect in the enzyme systems of the pineal gland (possibly a defect of O-methyl transferase) there is an abnormality in the metabolism of serotonin. Consequently, the production of melatonin is reduced or absent with the formation of

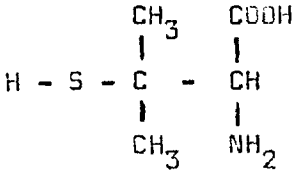
Fig. 2. Structural Formulae of Methionine, Cysteine, Penicillamine and Methionine Sulphoximine.



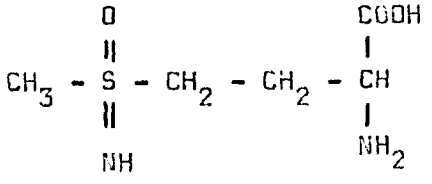
METHIONINE.



CYSTEINE



PENICILLAMINE



METHIONINE SULPHOXIMINE

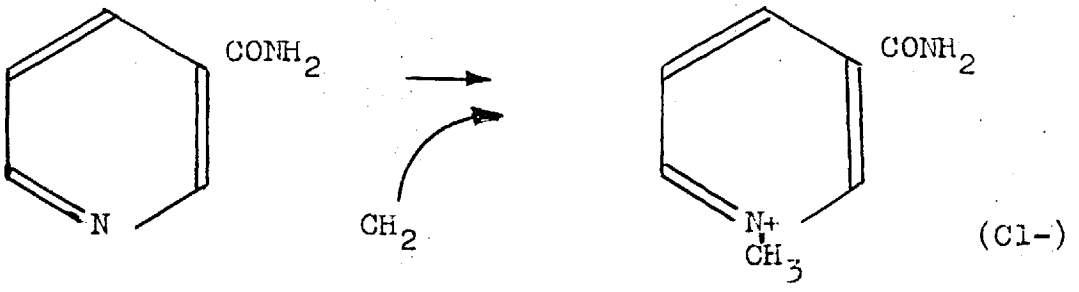
an abnormal pathway leading to the production of a psychotogenic substance (probably chemically similar to harmaline). As a result of the decrease in melatonin formation there is increased melanogenesis; and due to the production of harmala alkaloids, there are hallucinations and delusions."

It has been suggested (as an alternative) that penicillamine might act by interfering with biochemical transformations involving transmethylation (Smythies, 1966), a suggestion based on the chemical similarity between penicillamine and methionine (see Fig. 2).

This is speculative, and further biochemical studies will be required to evaluate the mode of action of penicillamine on transmethylation. As far as clinical effects are considered, mention may be made of a "preliminary" report by Helmchen et al. (1967) that d-penicillamine was of value in five schizophrenics, improving their affective and volitional status.

On the other hand there have been reports that penicillamine is of no value in acute schizophrenia (Affleck et al., 1969) or in chronic schizophrenia (Affleck et al., 1969; Brooker et al., 1968; Hollister et al., 1966 and Walshe, 1967). In all these studies, unlike that of Nicolson et al. (1966), dietary copper was not restricted. That this is unlikely to be important is suggested by the fact that beneficial effects noted in Nicolson's study occurred too quickly (within a matter of days) to be dependent on dietary

Figure 3. Methyl acceptance by Nicotinamide.



Nicotinamide

N^1-methyl Nicotinamide (Chloride)
(N.M.N.)

copper withdrawal.

g) Methyl acceptors as treatment for schizophrenia.

In introducing this topic, one might, perhaps, draw attention to an historical aspect of the subject : Hoffer (who has worked with Osmond, an originator with Smythies in 1952 of the trans-methylation hypothesis) wrote (1967) - "At our first internal research meeting early in 1952 we determined to use sufficient nicotinic acid (or amide) to decrease the transmethylation of noradrenaline to adrenaline....." Thus the rationale for the use of nicotinamide in schizophrenia was a consequence of the faulty transmethylation hypothesis.

Methyl acceptance takes place as shown in Figure 3.

Hoffer (1967) summarised the positive results of his earlier work and concluded that "When nicotinamide or nicotinic acid at a dose of 3 G/day is included in a treatment program for schizophrenia patients of the kind normally admitted to psychiatric wards of general hospitals, the therapeutic results are better than when it is not given." His results appeared to be the same whether nicotinamide or nicotinic acid was used; the latter produces a marked flush which makes "blind" evaluation of the effect of the drug impossible. Hoffer's earlier work, in which either nicotinamide (which does not produce a flush) or placebo were given (allocation to a 30 day course of therapy being at random) is more

acceptable from the experimental point of view than his later reports. In his earlier study,clinical comparison of the nicotinamide group with the placebo group showed that the vitamin treated group had done better than the placebo group (p= 0.039 by a Fisher Irwin test : author's calculation).

Hoffer has claimed improvement to occur more often in the acute than in the more chronic type of patient.

There has recently been a resurgence of interest in medication with nicotinic acid, nicotinamide and other vitamins in schizophrenia and in other psychiatric conditions. Megavitamin therapy has been widely acclaimed, particularly in the United States (and has received some support from a most eminent biochemist - Linus Pauling, 1968). Maslowski (1967) reported improvement in 17 of 30 male drug refactory schizophrenics given nicotinic acid; Gard (1967) reported improvement on either nicotinic acid or nicotinamide (coupled with conventional therapy and often with vitamin C) in both schizophrenic and non-schizophrenic psychiatric patients; Herjanic et al. (1967) reported that a series of schizophrenic outpatients did better on nicotinic acid than did a control group not so treated but that chronic hospitalised schizophrenics did not improve on this treatment relative to a control group.

Hoffer's claim that nicotinamide adenine dinucleotide

(N.A.D. otherwise known as D.P.N.) is an effective treatment for acute and chronic schizophrenic does not seem to have been substantiated. Kline et al., (1967) in a carefully controlled study found no difference in effect between the administration of D.P.N. and the administration of placebo on the clinical state of chronic schizophrenics, nor did Gallant et al. (1966). Hoffer and Osmond (1968) criticised Kline's work on the grounds that Kline's patients were more chronic than theirs; that Kline's N.A.D. was less active than theirs and that Kline's statistics did show some improvement to have occurred.

It is known by in vitro studies in animals that the administration of nicotinamide causes an elevation in N.A.D. levels. If, and the evidence is far from convincing, nicotinamide is of benefit in schizophrenia, the mode of action of the nicotinamide cannot (because of the disagreement of the efficacy of N.A.D.), be taken as proven to lie in its property/being an N.A.D. precursor.

In summarising the present situation it would seem reasonable to agree with Mayer (1969), that there is a need (in assessing the efficacy of nicotinamide in schizophrenia) for systemic experimentation using "tight diagnostic criteria and well defined methodological precautions such as double blind techniques." As has already been pointed out this is scarcely possible when nicotinic acid is administered, because of the flush it produces.

N Homocystinuria and Schizophrenia.

Homocystinuria is an inborn error of methionine metabolism controlled by an autosomal gene having a recessive character. Methionine can be converted in the body to homocysteine; thence, in the normal subject, cystathionine synthetase converts homocysteine to cystathionine and from this amino acid cysteine, cystine, taurine and ultimately inorganic sulphate are produced. In homocystinuria, the enzyme cystathionine synthetase is deficient and the methionine-cystine pathway is blocked at this point (see Fig. 1, p 8).

Homocystinuria is characterised by the excretion of excessive quantities of homocystine in the urine and by elevation of homocystine and methionine levels in the blood. The methionine pool is increased (Brenton and Cusworth, 1966). Patients suffering from this condition have a general appearance reminiscent of Marfan's Syndrome but show mental retardation and thrombotic phenomena, manifestations not encountered in the latter.

The interest of homocystinuria to the investigator into schizophrenic biochemistry lies in observations of schizophrenia in the families of homocystinurics by Carson et al. (1963), White et al. (1964) and Carey et al. (1968). Schimke et al. (1965) reported mental illness (the nature of which was unspecified) and suicide to be increased in homocystinurics' families. Spiro et al. (1965)

described a 24 year old woman who had both homocystinura and schizophrenia; her mother was considered to be an "ambulatory schizophrenic." Price et al. (1960) have drawn attention to an abnormal tortuosity in the nail fold capillaries together with an increase in the subpapillary plexus in a homocystinuric and also in his non-homocystinuric non-schizophrenic father. Similar changes have been described in a number of papers by R. R. Maricq as occurring in the nail folds of schizophrenics (see Maricq, 1966), such shizophrenics having a relatively high family incidence of the disease.

One could reasonably formulate the hypothesis that the predisposition to schizophrenia is increased in individuals who are homocystinuria heterozygotes : such a hypothesis would be consistent with those pedigrees published to date in which both homocystinuria and schizophrenia occur.

Biochemical studies on heterozygotes (parents of homocystinuric offspring) have been limited but Brenton, Cusworth and Gaull (1965) found, after giving an oral dose of methionine to the father of a homocystinuric that the serum methionine level had not returned to a basal level after 24 hours, as normally occurs. Carson et al. (1963) administered L-methionine orally to the mother (a paranoid schizophrenic) of two homocystinuric children and found that urinary methionine rose to higher levels than occurred with

normal subjects (consistent with the presence in this individual also of above normal serum methionine levels). On theoretical grounds (because methionine can be metabolised in other ways than via the cystine pathway - for example by incorporation into protein, and because the heterozygote should have cystathionine synthetase activity of a substantial degree, i.e. 50% of normal), one would judge methionine feeding likely to prove too crude a method to detect the homocystinuria heterozygote reliably and it is not surprising that some presumed heterozygotes have given normal results using such methods.

If one were to take individuals A and B carrying a gene (or genes) for schizophrenia and specify that one of these (A) was in addition a homocystinuric heterozygote, then individual A would tend to maintain the higher serum methionine levels; this, from what has been said concerning the exacerbation of schizophrenia by methionine (and M.A.O.I.), should tend to make A likely to show more schizophrenic disturbance than that shown by B, other things being equal. In genetic terminology, homocystinuria heterozygosity could tend to increase the expressivity of the 'schizophrenic' gene(s).

As far as the transmethylation hypothesis is concerned, doubt as to the precise mode of action of the methionine/M.A.O.I. combination in the feeding studies already discussed applies here also (see p 11).

The offspring of schizophrenic couples. One might anticipate that a study of the offspring of marriages between two schizophrenics would readily support or refute the hypothesis that some schizophrenics are homocystinuric heterozygotes, because such marriages should lead to homocystinuria among offspring at a frequency higher than that in the general population.

Further consideration shows that this is not the case. Thus if 1 in N schizophrenics is a homocystinuria heterozygote then, with random mating, 1 in N^2 marriages between schizophrenic couples will bring two homocystinuria heterozygotes together. (This value for N^2 will be higher than the true figure because of consanguineous marriage). One in $4N^2$ of the children of such marriages will be homocystinuric. Thus 1 in $4N^2$ such marriages will be expected to yield only 2 - 3 homocystinurics (assuming average fertility for such marriages); if N = 10 (which represents a substantial genetic contribution to schizophrenia from the abnormal gene in question), it will require 400 such marriages to produce this number of homocystinuric children.

Rosenthal (1966) in a study of the offspring of schizophrenic couples points out that the schizophrenia in his patients was atypical in that the average age of onset was a decade later than the average age of onset of schizophrenia : these individuals were able to marry, conceive and bear children before the onset of their illness.

However, Erlenmeyer-Kimling (1968) has suggested that the "late onset" in schizophrenic matings may be an artefact, early onset schizophrenia predisposing to family disruption and difficulty in ascertainment, particularly where both parents are affected.

If it is shown that homocystinuria heterozygosity predisposes to early onset rather than late onset schizophrenia (and one would expect that a genetic loading, if relevant, would act in this direction) the chance of a homocystinuria heterozygote schizophrenic marrying could be lower than the average for all schizophrenics.

The published data from five sources summarised by Rosenthal (1966) give information on only 67 schizophrenic couples. Many of their offspring died in infancy and early childhood (as many homocystinurics do), but homocystinuria had not been described when these series were collected; furthermore, the various authors were looking primarily at the incidence of schizophrenia in middle aged offspring. Even if homocystinurics had been present in the families, such individuals would not have been presented for study, for homocystinuria does not appear to carry much chance of survival beyond the age of 25.

To summarise: the present situation is one in which there is insufficient data on which to draw valid conclusions, for a sufficiently large survey of homocystinuric pedigrees has not

yet been assembled. The co-existence in pedigrees (of homocystinuria and schizophrenia) so far reported may be coincidental and spuriously impressive because pedigrees lacking such an association may not have been published. If such an association were established, the biochemical abnormality in the homocystinuria heterozygote would be worthy of very detailed study. The nature of this abnormality might support a "fauly transmethylation" hypothesis. Alternatively it might suggest new avenues of approach to the problem of the biochemical lesion in schizophrenia.

Studies to date on schizophrenic couples have not reported any increase of homocystinuria among their offspring. For a host of reasons this is not surprising. It certainly does not invalidate the hypothesis that the homocystinuria heterozygote may carry an increased risk of developing schizophrenia.

i) Catechol-O-Methyltransferase (C.O.M.T.) administration to Schizophrenics.

Hall et al. (1969) reported the results of administering intramuscular injections of C.O.M.T. (obtained from etiolated peas) to four moderately disturbed schizophrenics who had not been treated with either phenothiazines or E.C.T. immediately prior to the injections which were given daily for 1 - 2 weeks.

All patients appeared to worsen on this therapy although in one case the symptoms recorded (dizziness, headache, insomnia and

depression) were not those of exacerbated schizophrenia. Clinical ratings were not carried out blind and no control subjects were studied; moreover the patients may have had some knowledge of what the investigators considered might happen since they gave "fully informed consent."

In one patient cessation of injections lead to improvement, a further course of injections then leading to further worsening of symptoms (and transiently to the appearance of a urinary 'pink spot').

This report, although of a preliminary nature, is of considerable interest. If the effects obtained are confirmed and shown to be specific to schizophrenics, they would provide evidence suggesting not only that a significant abnormal pathway in the schizophrenic may contain a methylation step, but also perhaps, that it is not this step that is abnormal. If it were, it is difficult to see why this particular enzyme should produce an adverse effect for by competing with the pathological enzyme for substrate and cofactors, it might not unreasonably be predicted to have, if anything, a beneficial effect.

j) The 'Pink Spot' Controversy.

In 1962 Friedhoff and Van Winkle reported the excretion of the compound 3:4 dimethoxyphenylethylamine (D.M.P.E.) in the urine of 15 out of 19 schizophrenics but in none of 14 normal subjects; their paper chromatographic and staining methods gave rise to a

'pink spot.' Since this time a large number of articles on this topic representing many different shades of opinion have appeared.

The present position is that there is more agreement about the presence of 'pink spot' material in the urine of schizophrenics than about its identity. The most likely reasons for the wide disagreements that exist in this field are:-

a) that not all series are concerned with the same type of schizophrenics (some concentrating on acute patients, others on chronic).

b) that techniques differ widely from series to series : all reported pink spots may not be produced from the same substance.

c) that some investigators may not satisfactorily have overcome the difficulties imposed by the presence of drug metabolites in urine obtained from schizophrenics.

d) that if 'pink spot' is not D.M.P.E. then studies designed to estimate the latter substance are likely to be irrelevant to the pink spot phenomenon.

As far as the excretion of D.M.P.E. itself is concerned, there is little general agreement. Sen and McGeer (1964) and Kuehl et al. (1964) have claimed to have demonstrated this compound in the urine of schizophrenics using gas chromatography and thin layer chromatography, respectively. Takesada et al. (1963) claimed that

it was present in the urine not only of their schizophrenics but also in 45% of their controls. Studnitz and Nyman (1965) and Vogel et al. (1967b) found D.M.P.E. to be excreted both by normals and schizophrenics while Barbeau et al. (1963) reported it in the urine of patients with Parkinson's disease (although this finding has been disputed by others).

On the other hand, Perry et al. (1964, 1966) failed to find D.M.P.E. in the urine of either acute or chronic schizophrenics, while Williams et al. (1966) and Jones et al. (1969) failed to find it in the urine of any of a number of acute schizophrenics.

Friedhoff and Van Winkle (1967) have continued to assert that the compound they have been studying is D.M.P.E. and thin layer and gas chromatography and melting point estimations have been used to back up this assertion. Creveling and Daly (1967) produced mass spectroscopic evidence that part of the paper chromatographic phenomenon of Friedhoff and Van Winkle is D.M.P.E. although they considered this compound to be only a minor constituent of the 'pink spot.' They subjected the pink spot area of unstained paper chromatograms to elution and then ran the eluate on T.L.C. with the production of 7 spots, in one of which they identified D.M.P.E. by preparing dansylamine derivatives which were then subjected to further chromatography and finally to mass spectroscopy analysis.

Other workers would accept that a chromatographic pink spot

can be derived from the urine of schizophrenics but would not agree that this is produced by D.M.P.E. Pind and Fauvye (1966) came to this conclusion, as did Bell and Somerville (1966) and Boulton and Felton (1966).

Alternative Identification of Pink Spot.

a) p-tyramine. Boulton et al. (1967) suggested that the pink spot may derive from p-tyramine. Vogel et al. (1967a) examined and rejected this possibility on the grounds that p-tyramine was excreted by all the subjects they tested, a group of schizophrenics excreting somewhat less, on average, than a control group. Furthermore, Takesada et al. (1963) showed that p-tyramine and D.M.P.E. have distinctly different Rfs on the butanol : acetic acid : water solvent system using which Friedhoff and Van Winkle (1962) originally reported D.M.P.E. and the substance yielding a pink spot to be isographic.

b) a chlorpromazine metabolite. Closs et al. (1967) made the tentative suggestion that pink spot might be produced from nor-chlorpromazine sulphoxide (a chlorpromazine metabolite) but Steinberg and Hudson (1966) in a study of this possibility concluded (on chemical evidence) that this substance could not be the material isolated by Friedhoff and Van Winkle. The findings of Ridges et al. (1968) of a group of pink spot positive cases in which drugs had on no occasion been given also argues against the

drug metabolite theory. Ridges and Harper (1968) could find no correlation between pink spot and the administration of one particular drug. Bourdillon et al. (1965) found that the relationship between schizophrenia and pink spot was at least as marked in those who have been off drugs for two weeks or more as in those who had not, but this is not entirely conclusive since phenothiazines and their metabolites may be excreted for many months after cessation of therapy (Goodman and Gilman, 1965).

c) tryptamine. Kuehl et al. (1968) argued that tryptamine is responsible for the 'pink spot' phenomenon, and in this connection it is of interest to note that Berlet et al. (1964) found peaks of tryptamine excretion to precede the peak of worsening of schizophrenic symptoms during exacerbations of this disease. However, Bourdillon and Ridges (1970, in press) have chromatographic evidence that the pink spot which they have been studying cannot be produced from tryptamine.

d) monopropionyl cadaverine. Perry et al. (1967) suggested that monopropionyl cadaverine produced the pink spot and that this arose either as the product of intestinal flora peculiar to life in an institution, or as a result of constipation. However, the technique used by several other workers was such that monopropionyl cadaverine would have been discarded and could not have explained their pink spots (Watt et al., 1969). Perry's suggestions are

also contrary to those of Bourdillon et al. (1965) who examined urine samples from 296 psychiatric inpatients, including many chronic patients. It was noted first, that the pink spot was found significantly more often in the schizophrenic than in the non-schizophrenic ($p<0.001$) notwithstanding the fact that both types of patient were living on the same 'chronic' wards, and second, that the incidence of pink spot in the chronic group was lower than in a group of patients newly admitted (and often acutely disturbed) also examined in this study. Furthermore, Ridges et al. (1968) found that pink spot production was unaffected by a 5-day course of neomycin which would considerably alter gut flora. All these points argue against the intestinal flora theory of pink spot origin.

Other suggestions as to the origin of the pink spot. Perry et al. (1967) suggested that their failure to find D.M.P.E. in the urine of schizophrenics may have been due to the fact that many amines in urine are derived from dietary plant sources. They examined urine obtained from patients maintained for five days on a diet free of all fruit, vegetables and flavouring agents, but failed to find D.M.P.E.. Von Studnitz and Nyman (1965) also considered D.M.P.E. to be of dietary origin because it disappeared from the urine after several days on a glucose diet both in schizophrenics and normal subjects (as is mentioned above these workers claimed

to find D.M.P.E. in urine from schizophrenics and from mentally healthy controls).

Friedhoff and Coupet (1967) pointed out that urinary homovanillic acid (H.V.A., a metabolite of dopamine) decreased sharply when a glucose diet was given although this acid is thought to be of endogenous origin. They suggested that this indicates that the argument that 'since D.M.P.E. disappears from the urine on a glucose free diet, therefore it derives from the diet' is a non sequitir. The objections put forward by Friedhoff and Coupet to the work of Von Studnitz and Nyman seems to the present writer to be illogical. What Friedhoff and Coupet showed was that urinary H.V.A. was likely to be derived in part from dietary sources (as well as from dopamine metabolism) and this is as far as their argument should have taken them. A more satisfactory question for them to have asked would be 'urinary D.M.P.E. might be derived from a dietary constituent but why only in schizophrenics?' Furthermore, a far more fundamental point of difference exists : that Von Studnitz has described D.M.P.E. as occurring in the urine of mentally normal subjects whereas Friedhoff and his co-workers have not (except in a twin study described on p 37).

This has relevance to the problem of pink spot and schizophrenia only if pink spot is D.M.P.E. and there is a substantial body of evidence that it is not, as has been indicated.

In this connection one might mention that Bourdillon et al. (1965) included non-schizophrenic patients from the same wards who had been offered the same diet as had the schizophrenics, suggesting that dietary factors alone afford an incomplete explanation of the pink spot phenomenon.

Nunn and Wheeler (1966) suggested that pink spot may result from brain damage. They described a leucotomized 69 year-old schizophrenic (previously treated with massive doses of phenothiazines) who showed considerable dyskinesia and whose urine yielded a pink spot. It is, however, clear from other studies that phenothiazine therapy, brain surgery and dyskinesia are not prerequisites for pink spot production.

Other considerations concerning the pink spot phenomenon.

The phase of original optimism concerning the pink spot phenomenon was followed by scepticism and highly critical attacks exemplified by a leading article in the British Medical Journal (1967), which seemed determined once and for all to dismiss the pink spot as yet another red herring. But a specific explanation of the phenomenon as being due to dietary or gut flora effects or to differences in bodily activity or to drug metabolites must account for such facts as the most methodologically acceptable experiments have established. It does not seem to the present author that such explanations as have been forward to date have

been successful in doing this.

The most carefully controlled experimentation to date was presented by Bourdillon et al. (1965) who found (in a series of experiments in which biochemical and clinical data were collected by independent workers) a highly significant correlation between schizophrenia and pink spot. This applied particularly for non-paranoid schizophrenics and for schizophrenics with Schneider-positive symptoms. (These are symptoms thought by Schneider, 1959, to be indicative of schizophrenia.) Substantial control groups of mentally normal individuals and non-schizophrenic mental hospital inpatients were provided. The degree of probability of an association between a diagnosis of schizophrenia and pink spot can be judged from the following table.

TABLE 1: Comparison of Incidence of Pink Spot between Classes.

(from Bourdillon et al. 1965)

	p
Schneider positive (46) with control (149)	$=2.5 \times 10^{-29}$
Schneider negative (23) with control (149)	$=3.1 \times 10^{-7}$
Schneider positive (46) with Schneider negative (23)	<0.001
Non-paranoid schizophrenic (69) with non-schizophrenic (77)	<0.001
Non-paranoid schizophrenic (52) with control (149)	$=8.06 \times 10^{-32}$

(The first member of each pair contains the higher proportion of pink spot positive subjects ; p is the probability coefficient. The figures in brackets are the numbers in each class).

A satisfactory explanation of these findings in biochemical terms has yet to be given, since Bourdillon did not attempt to equate D.M.P.E. and pink spot.

Predescu et al. (1968) carried out a similar study to that of Bourdillon et al. (1965) (although not on a double blind basis) using the technique of Friedhoff and Van Winkle. They derived 23 pink spots from the urine of 51 schizophrenics (who had all been off treatment for at least three weeks). Urine from 170 non-schizophrenic subjects (55 mental hospital patients, 88 subjects ill with physical illness and 27 healthy subjects) yielded only one pink spot.

To summarise, the pink spot phenomenon would appear to stand up fairly well to critical examination as a phenomenon peculiar to schizophrenia. Explanations of pink spot as being some form of artefact have as yet failed satisfactorily to account for the known facts. D.M.P.E. seems very unlikely to be wholly responsible for the pink spot. An alternative identity has yet to be established; more than one compound may be responsible for the phenomenon in any given instance.

The relationship between the transmethylation hypothesis and the pink spot phenomenon. Until the substance(s) responsible for the pink spot phenomenon has been identified this relationship remains conjectural. The phenomenon may yet prove to afford an important

source of support for such a hypothesis. It is to be hoped that further mass spectroscopy studies similar to that of Creveling and Daly (1967) (see p 29) will be carried out.

Studies of urinary D.M.P.E. in MZ twinships discordant for schizophrenia. It is of interest that Friedhoff has recently claimed to have found D.M.P.E. in the urine of non-schizophrenic co-twins of several monozygotic twin pairs discordant for schizophrenia (see Stabenau et al., 1969). Since Friedhoff (1967) had previously found D.M.P.E. only in schizophrenic individuals, this finding raises several important issues which will require further elucidation among which is the hypothesis that D.M.P.E. excretion is related primarily to a certain genetic constitution. A further point of considerable interest is whether schizophrenia will develop in the 4 (out of a total of 6) non-schizophrenia co-twins in which D.M.P.E. was found (and not in the others).

This assumes that D.M.P.E. is what Friedhoff has estimated in the urine samples, an assumption open to dispute. If the above findings can be substantiated, those non-schizophrenic co-twins who are excreting a 'pink spot' producing substance would be an excellent source of urine for further analysis, since they do not require phenothiazines as schizophrenics do. These drugs can both decrease and mask pink spot production (Ridges and Bourdillon, 1967; Bourdillon et al., 1965).

D.M.P.E. and Homocystinuria. Welch et al., (1969) could find no D.M.P.E. in urine from homocystinurics or homocystinuria heterozygotes.

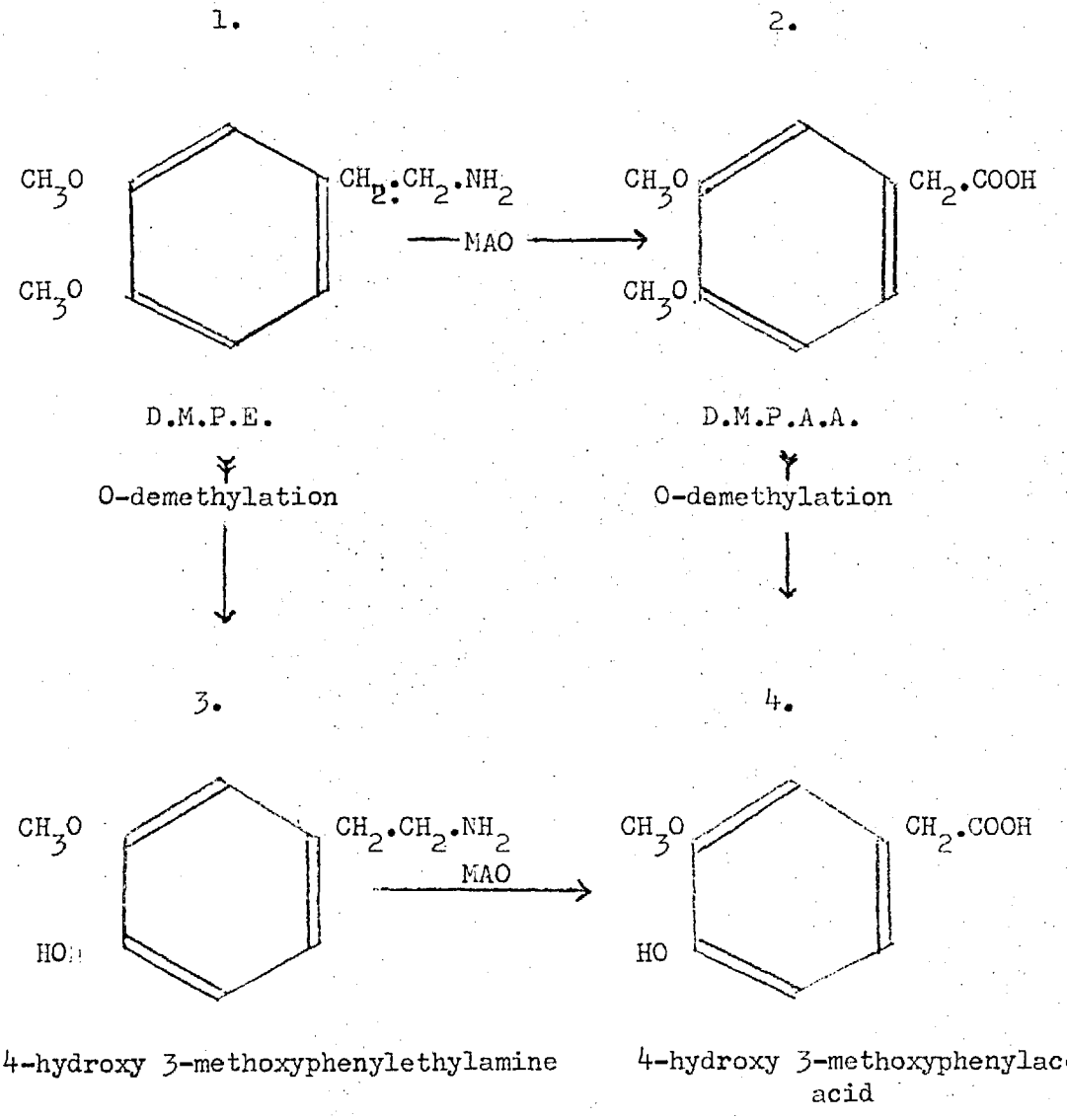
A note on the pharmacological properties of D.M.P.E.

It would be out of place to devote much space to an account of the pharmacological properties of D.M.P.E., since there is little support for the assertion that it is the compound chiefly responsible for the pink spot phenomenon.

D.M.P.E. has not proved to be a psychotomimetic when given to human volunteers (Shulgin et al., 1966; Hollister and Friedhoff, 1966; Charalampous and Tansey, 1967) in doses of up to 1 gram (except that Brown et al., 1968, described one normal subject who responded to an oral dose of 5 mgm./Kg. with a typical mescaline like response lasting three hours which could not be reproduced subsequently even when a much higher dosage was used; the authors favoured the initial positive reponse as representing a "chance placebo occurence").

Administration to humans has been by the oral route. This exposes the compound to gut and liver amine oxidase. In studies on the compound in experimental animals larger doses, by a parenteral route have usually been used. Vogel (1968) has suggested that further experiments with D.M.P.E. in human volunteers need to be carried out in which plasma levels should be measured to establish whether or not it is likely that brain levels found critical in animals (i.e. for a behavioural response) can be achieved in man.

Figure 4. The Catabolism of administered D.M.P.E.



4-hydroxy 3-methoxyphenylethylamine

4-hydroxy 3-methoxyphenylacetic acid

Compounds 1,2 &4 are urinary metabolites of D.M.P.E. in man.
(Charalampous & Tansey , 1967)

The Catabolism of D.M.P.E.

Absorption of oral D.M.P.E. in man appears to be both rapid and virtually complete (Charalampous and Tansey, 1967).

Studies of D.M.P.E. catabolism are in general agreement that 3:4 dimethoxyphenylacetic acid (D.M.P.A.A.) is the main metabolite of D.M.P.E. in man (Friedhoff and Hollister, 1966; Schweitzer and Friedhoff, 1968 and Charalampous and Tansey, 1967). The 4-O demethylated derivative of 3:4 dimethoxyphenylacetic acid, 4-hydroxy 3-methoxy phenylacetic acid (H.M.P.A.A.) was shown to be a D.M.P.E. metabolite by Charalampous and Tansey (1967). These authors found the following percentages for the various metabolites in a 12 hour urine collection after an oral dose of 600 mgm. of D.M.P.E. : D.M.P.A.A. 90.6; H.M.P.A.A. 1.4; D.M.P.E. 0.2. There were a further seven unidentified metabolites. In this study (in which the 600 mgm. dose carried a small amount of C^{14} D.M.P.E.) N-acetyl derivatives and the demethylated amine 4-hydroxy 3-methoxy phenylethylamine were sought unsuccessfully.

H.M.P.A.A. might derive from D.M.P.E. via one or both of the two routes shown in Fig. 4 . Charalampous and Tansey (1967) established the existence of the pathway via D.M.P.A.A. in man, by showing H.M.P.A.A. to be a metabolite of administered D.M.P.A.A. (having also shown that D.M.P.A.A. could be derived from D.M.P.E., as mentioned above).

Urinary D.M.P.A.A.

The chief metabolite of administered D.M.P.E., 3:4 dimethoxyphenylacetic acid (D.M.P.A.A.) has been reported to be a normal urinary constituent both by Kuehl et al. (1966) and Friedhoff and Furiya (1967). Neither Kuehl et al. (1966) nor Vogel et al. (1967b) found any significant difference in the excretion of D.M.P.A.A. between schizophrenics and normal subjects. The interpretation put by Kuehl et al. (1966) on this finding is that either urinary D.M.P.E. is not characteristic of schizophrenia or that the amount of D.M.P.A.A. is not a true reflection of the amount of D.M.P.E. being excreted. That the latter is not unlikely is supported by work done by the author (Price, 1969) who has shown that the homologue of D.M.P.A.A., 3:4 dimethoxybenzoic acid (veratric acid, V.R.A.) is a minor metabolite of vanillic acid which is itself a metabolite of caffeic acid present in coffeee, cocoa and some fruit. In other words, a pathway exists whereby dietary phenolic acids can be converted to 3:4 dimethoxyphenolic acids; the small amounts of D.M.P.A.A. found in urine (Friedhoff and Furiya found 25 - 500 μG/24 hours) may derive, in part, from dietary phenolic acids. Alternatively, methylation of endogenous phenolic acids (for example of homovanillic acid to yield D.M.P.A.A.) may occur.

k) Methylation by Schizophrenics and Non-Schizophrenics in vivo and in vitro.

The assertion that D.M.P.E. was present in the urine of schizophrenics provided the stimulus for several studies on the methylation of dopamine, both in vitro and in vivo. There is a good reason why dopamine is not a good substrate for studies of methylation processes : it has too many metabolites. Thus Goodall and Alton (1968) in a study of labelled dopamine given intravenously to human subjects listed data on 11 unconjugated metabolites, 9 conjugates and 7 unknown metabolites. Amine oxidation, O-methylation, conversion to noradrenaline (and its metabolites) and conversion to ethanolic and glycolic metabolites were all noted to occur. In such a complex situation the wisdom of attempting to assess O-methylation using dopamine as substrate would seem open to question.

Friedhoff and Van Winkle (1963) administered H^3 labelled dopamine intravenously to five hospitalised schizophrenics whom they had previously considered to be excreting D.M.P.E. in the urine. The urine subsequently collected was claimed to contain labelled D.M.P.A.A. In a further experiment liver biopsy material was obtained from one of these schizophrenics; a homogenate of this was incubated with tritiated dopamine (H^3), magnesium chloride and C^{14} methyl labelled S-adenosyl-methionine. Material

subsequently eluted from a part of the chromatogram corresponding to 3:4 dimethoxyphenylacetic acid had a C^{14}/H^{3} ratio of very nearly 2, suggesting that a labelled methyl group had attached itself to both 3: and 4: positions. In these studies no control subjects were studied.

Wagner et al. (1966) reported that two chronic schizophrenics given labelled dihydroxyphenylalanine (D.O.P.A.) failed to convert it either to D.M.P.E. or to D.M.P.A.A.

Faurbye and Pind (1967) followed the metabolism of labelled dopamine in vivo in one normal subject and four chronic schizophrenics. They subsequently examined urine for a number of possible metabolites - D.M.P.E., D.M.P.A.A. and five other metabolites (these only accounted in all for about 20% of the radioactivity in the urine, indicating an incomplete analysis of the situation). They were unable to detect D.M.P.E. in urine from any of their five subjects or to find any significant differences between the normal subject and the schizophrenics in the excretion of those dopamine metabolites which they measured. Minute amounts of radioactivity were found in the D.M.P.A.A. spots (unlabelled carrier D.M.P.A.A. had been added to the urine at the beginning of the analysis). This was present in urine from the normal subject and three of the four schizophrenics; it was suggested that it arose from 4-O-methylation of H.V.A.

Mono-methylation in the 4- position was not considered separately in this study.

Kuehl (1967) carried out studies on the formation of D.M.P.E. from labelled dopamine. His experiments were 'in vitro,' and employed human autopsy material.

Estimates of the labelled mono-O-methyldopamine (a mixture of 3 methoxy-4-hydroxy- and 4 methoxy-3-hydroxy-phenylethylamine) and of D.M.P.E. were subsequently made. Kuehl's results are shown in Tables II and III.

Table II. Results of in vitro studies with human liver (from Kuehl, 1967).

Human Liver Diagnosis	Age	Sex	Death-autopsy interval	Relative C.O.M.T. activity*	D.M.P.E. (c.p.m.)
N	2	-	5 hr	1.00	844
N	26	F	4 hr	0.48	498
N	-	-	3 hr	0.91	81
S	-	-		0.005	41
S	-	-		0.003	0
S	77	F	40 hr	0.39	38
S	78	F	8.5 hr	0.05	36
S	75	M	6 days	0.01	7
S	89	F	3 days	0.25	21

N = Normal S = Schizophrenic

* derived from c.p.m. of Mono-O-methyldopamine (range 3,269 - 1,057,820).

Table III: Results of in vitro studies with human brain (From Kuehl, 1967).

Human Brain.

Diagnosis	Age	Sex	Death-autopsy interval	Relative C.O.M.T. activity	D.M.P.E. (c.p.m.)
S	34	M	6 -8 hrs	2.06	24
N	72	M	"	1.00	11
S	74	M	"	0.19	8
S	-	M	"	3.02	36
S	48	M	"	0.50	17

N = Normal S = Schizophrenic.

Kuehl evidently regarded the counts for D.M.P.E. to be so low as to be "negative," but further consideration of the data reveals that a correlation does exist between the yields of mon-O-methyl dopamine and D.M.P.E.

Results for Liver: If logs are taken of the figures given in Table II for relative activity of C.O.M.T. (multiplied by 10^3 for convenience) and the counts per minute for D.M.P.E. (increasing the value of the D.M.P.E. count for liver 5 from 0 to 1 so as to avoid the weighting of minus infinity for $Log_{10}0$), then the values obtained are those set out in Table IV.

Table IV: Data from Table II treated logarithmically.

Relative C.O.M.T. activity	O.M.P.E.
3.0000	2.9263
2.6812	2.6972
2.9590	1.9085
0.6990	1.6128
0.4771	0.0000
2.5911	1.5798
1.6990	1.5563
1.0000	0.8451
2.3979	1.3222

The correlation coefficient for these two sets of figures is $r = +0.7696$ with $t = 3.1885$ and $0.01 > p > 0.001$.

Results for Brain: A direct comparison between Relative C.O.M.T. activity and O.M.P.E. (c.p.m.) gives $r = 0.9369$, $t = 4.6426$ and $0.01 > p > 0.001$.

Thus a relationship between C.O.M.T. activity and O.M.P.E. production can be seen to exist in this data. Comparison between the absolute rates of production of methylated derivates of dopamine using the data from liver cannot be made because the interval between post mortem and autopsy averaged four hours for the three normals, whereas the intervals for the four schizophrenics

about whom this information was given were 8.5 hours, 40 hours, 3 days and 6 days. The intervals between death and autopsy for schizophrenics are so long that the value of experiments carried out on such necrotic material must surely be highly questionable.

In the data obtained from brain, the interval between death and autopsy was 6-8 hours in each case. It is to be noted that there is a 16-fold difference in C.O.M.T. activity between two of the schizophrenics studied and this suggests that direct comparisons between the activities obtained from the five brains should not be made. Thus the most important factor determining such a major difference in enzyme activity would seem likely to have been the amount of post-mortem change occurring in the brains of the various individuals during the considerable interval between death and autopsy.

In Table V, the results of the various experiments on the synthesis of D.M.P.E. and D.M.P.A.A. from dopamine and D.O.P.A. are summarised.

TABLE V:

Dopamine and D.O.P.A. Metabolism in Non-schizophrenic and Schizophrenic Subjects.

	Substrate	Tissue	Non-schizophrenics		Schizophrenics	
			DMPE	DMPAA	DMPE	DMPAA
In vitro						
Friedhoff & Van Winkle (1963)	Dopamine	Liver (biopsy)	0	0	+	+
Kuehl (1967)	"	Liver (autopsy)	+?*	0	+?*	0
		Brain (autopsy)	+?*	0	+?*	0
In vivo						
Friedhoff & Van Winkle (1963)	Dopamine		0	0	+	+
Faurbye & Pind (1967)	"		-	+?	-	+?
Wagner et al. (1966)	D.O.P.A.		0	0	-	-

Key: 0 = not sought +? = presence in doubt

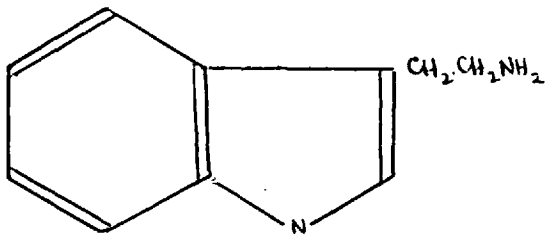
\+ = present - = absent

* Kuehl (1967) deduced that his results showed that D.M.P.E. was not formed from dopamine. This deduction is open to question (see p 45).

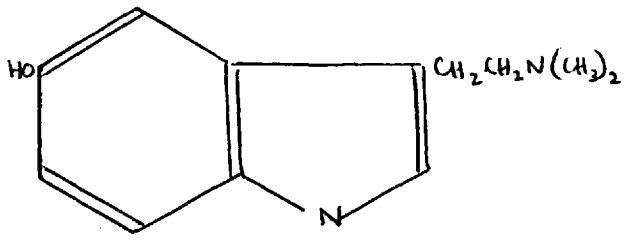
To summarise: from none of this experimental evidence can one deduce that schizophrenics can synthesize D.M.P.E. from dopamine and controls cannot.

1) Faulty transmethylation and tryptophan metabolism.

The particular topic to be discussed here is the occurrence of an N-methylated tryptamine derivative, Bufotenin, in the urine of schizophrenics. Since abnormalities of the metabolism of tryptamine itself have been described in schizophrenia, a brief discussion of this subject will also be included here.



Tryptamine



Bufotenin

A pathway exists in mammalian tissue whereby methylated indoleamines can be produced (Axelrod, 1961), rabbit lung containing an enzyme capable of the following transformations:-

Serotin ⟶ N-methylserotonin ⟶ Bufotenin

Tryptamine ⟶ N-methyltryptamine ⟶ Dimethyltryptamine

Axelrod found S-adenosyl methionine (which derives from methionine) to be a cofactor for this rabbit-lung enzyme.

a) Bufotenin in urine. Bufotenin is 5 hydroxy N:N dimethyl

tryptamine. Dimethyl tryptamine (D.M.T.) is a true hallucinogen but the 5-hydroxy derivative produces far more autonomic disturbance than D.M.T. does.

Fischer et al. (1961) reported bufotenin in urine from 25 out of 26 schizophrenics and from none of 15 non-psychotic patients. Among others, Rodnight (1961) and Sprince et al. (1963) were unable to confirm these findings.

A controversy has developed which more recent work has done little to resolve. Gross and Franken (1964) claimed to have found bufotenin in urine from normal subjects in amounts of 62.75 ± 7.2 μG/24 hr., while Perry et al. (1962) reported that small amounts of bufotenin were present in the urine of normal children. On the other hand, Takesada et al. (1965) using a method claimed to be able to detect 20 μG of bufotenin in a 24 hour collection failed to find it in the urine of normal subjects or of schizophrenics (the latter having "5 - 7 year courses of the disease").

Siegel (1965) described a method which was sensitive "to at least 1 μG of bufotenin per litre" and failed to find this amount in urine from any of five normal subjects and 21 schizophrenics, four of whom were hallucinating at the time of urine collection. Siegel regarded Fischer's paper chromatography method as crude, insensitive and incapable of producing quantitative results. He

suggested that Fischer's findings (of 400 μG/L of bufotenin in the urine of schozphrenics) resulted from those methodological inadequacies and from the fact that interfering substances diffused into the bufotenin area of the chromatogram when Fischer's methods were used. Siegel also regarded Gross and Franken's methods (1964) as suspect.

Runge et al. (1966) failed to find bufotenin in the urine of any of 22 chronic schizophrenics; they found that phenothiazines produced a blue spot in the bufotenin zone of their paper chromatograms and suggested that differences between their findings and those of Fischer may have been due to the fact that Fischer's patients were not only off drugs, but were also actively hallucinating at the time that their urine was examined.

Fischer and Spatz (1967), still using methods which seem relatively crude, reported bufotenin in 26 of 45 schizophrenics many of whom were early cases on no treatment. Their 49 controls included patients with epilepsy, oligophrenia, alcoholism and hysteria. All these controls failed to show urinary bufotenin. However, a more recent publication by the same authors (Fischer and Spatz, 1968) described a quantitative method using which non-schizophrenics were estimated to excrete 1.2 - 8.9 μG% (mean 3.6 μG%) and newly admitted schizophrenics to excrete usually more than 10 μG% (maximum 37.5 μG%, mean 17.2 μG%). It will be noted that

when these values are scaled up to correspond to 24 hour collections they represent amounts considerably greater than those described by most other workers who have claimed to have found bufotenin in urine.

Tanimukai et al. (1967b), employing sensitive methods, report conjugated bufotenin in four out of four mental defectives and six out of six chronic schizophrenics (all off treatment for at least seven weeks); free bufotenin was present in urine from all six schizophrenics but in urine from only one of the four mental defectives, this patient developing 'a psychotic reaction with ideas of reference' when he was transferred from the open ward to a closed ward for the investigation. Total bufotenin was estimated as being less than 1 μG per 24 hours. Faurbye and Pind (1968) found six normal subjects to excrete 0 - 7.5 μG of bufotenin/24 hours and seven chronic schizophrenics with active symptoms to excrete 0 - 3.7 μG of bufotenin/24 hours.

Brune (1965) and Tanimukai et al. (1967a) all working with Himwich, have repeatedly found bufotenin in the urine of schizophrenics. Their more recent work employs the more sensitive methods of thin layer and gas-liquid chromatography. They have found psychotic symptoms to worsen about two weeks after an increase in urinary bufotenin, with the persistence of a clinical exacerbation as long as increased urinary bufotenin has continued.

For these experiments urine from chronic schizophrenics has been examined with or without the prior administration to the patients either of an M.A.O.I. or of large amounts of an amino-acid (one of the following: methionine, betaine or cysteine), or of an M.A.O.I./amino acid combination. Bufotenin has been found in amounts of 4-10 μG/24 hours when a combination of therapy, including an M.A.O.I. has been administered.

Heller (1966) found that an M.A.O.I. given to chronic schizophrenics exacerbated symptoms and led to a bufotenin-like spot appearing on paper chromatograms. Acebal and Spatz (1967), on the other hand, claimed to have detected urinary bufotenin in seven out of 10 schizophrenics and showed that trifluperidol led both to a marked clinical improvement and to the disappearance of urinary bufotenin.

To summarise: There are so many discrepancies in these findings that no definite conclusion can yet be drawn. If Fischer is correct in his claims concerning the amounts of bufotenin excreted by schizophrenic and normal subjects, it is very strange that such large amounts should have eluded other investigators so consistently. The abandonment of relatively unsophisticated and insensitive methods of searching for bufotenin is clearly needed. Tanimukai's suggestion (1967b) of a correlation between psychotic symptoms and free bufotenin in the urine deserves

independent confirmation using methods as sensitive as those which he has developed.

b) Tryptamine in urine. Berlet et al. (1964) found a direct relationship between urinary tryptamine and the degree of psychotic activity in a number of male chronic schizophrenics off treatment; thus severely psychotic patients had higher urinary tryptamine levels than patients in a less psychotic phase. When tryptamine is acted upon by monoamine oxidase, 3-indoleacetic acid is produced. In Berlet's studies rising urinary tryptamine was paralleled by a rise in urinary 3-indoleacetic acid, indicating that the increased tryptaminuria was not the result of lowered monoamine oxidase activity.

Increase in the amount of urinary tryptamine is not specifically related to schizophrenia, the urinary level of this amine rising sixfold when M.A.O.I. therapy is administered to non-schizophrenic subjects (Sjoerdsma et al., 1960). However M.A.O.I.s tend to worsen schizophrenia, particularly in the acute phase, and may even precipitate the psychosis (Price and Hopkinson, 1968, have summarised the evidence relating to this subject). This is a drug-produced situation in which increased tryptaminuria and exacerbated schizophrenia may coexist.

The phenomenon of increased tryptaminuria during exacerbations of schizophrenia requires much more attention (but not in a

discussion of the faulty transmethylation hypothesis since tryptamine is not a methylated amine). It has been introduced at the present stage because bufotenin can derive from tryptamine (by hydroxylation and methylation), and it could be speculated that such increases in urinary bufotenin as may occur reflect a primary increase in tryptamine formation (or might result from the cause of that increase) rather than indicate overactivity in the methylation pathways described by Axelrod (1961).

Concluding observations on the faulty transmethylation hypothesis.

In general, the faulty transmethylation hypothesis receives support from evidence which is either disputed, unconfirmed or capable of an alternative explanation. This statement is not intended as a criticism of the hypothesis but rather to draw attention to the present need for further studies designed to resolve disputes (whether of a biochemical or phenomenological nature), to confirm interesting but unsubstantiated observations (such as the studies on methionine sulphoximine, on the effects of C.C.M.T. and on the metabolism of tagged methionine) and to elucidate the mechanism by which effects are produced (as in the aminoacid feeding studies).

Throughout this field there is a dearth of quantitive studies in which control subjects and schizophrenics have been compared in sizeable numbers in an unbiased way and in which proper

allowance has been made for complications introduced by the use of psychotropic drugs.

Smythies (1968) wrote "the transmethylation processes of schizophrenics need to be explored by any means which suggest themselves;" this rightly emphasises the need for further research into an approach, which, in spite of dispute, continues to appear promising.

Genetics and a Biochemical Basis for Schizophrenia.

The biochemical approach to schizophrenia adopted here would be attacked by some as being entirely unreasonable, for there are many theories of schizophrenia in which environmental factors are regarded as paramount.

In any disease which is to some extent genetically determined, a biochemical abnormality may be presumed to be present (although techniques in present use may be much too unsophisticated to demonstrate it). Where there is a multifactorial inheritance, a biochemical abnormality may be detectable even though each individual gene involved contributes only a small, and at present undetectable, effect (diabetes mellitus may exemplify this situation). Otherwise the concept originated by Garrod can be expected to apply : that an abnormal gene determines the occurrence of an imperfectly functioning or non-functioning enzyme, hence the biochemical abnormality.

It is, therefore, relevant to the biochemical approach to summarise the present views on the genetics of schizophrenia and to indicate the influence that genetic principles have had on the experimental approach adopted in the present work.

(i) The genetic basis of schizophrenia. That schizophrenia has a genetic basis seems to be generally accepted, in spite of Tiernari's assertion (1963) that 16 pairs of monozygotic twins in Finland were all discordant for the disease. Prior to the publication of

Tienari's work, the earlier work (e.g. that of Kallmann and Slater) was widely accepted as indicating a genetic basis for schizophrenia : since Tienari's publication, both Shield's (1965) and Slater (1967) have attacked Tienari's work on methodological and diagnostic grounds. Furthermore, Gottesman and Shields (1966), Kringlen (1967) and Fischer et al(1969) have published reports showing higher concordance rates for schizophrenia in monozygotic (MZ) than in dizygotic (DZ) twins, although concordance rates for MZ twins in more recent studies have been somewhat lower than the figures of 60 - 70% published earlier. There are a number of possible reasons for this but the most likely would seem to be that the earlier studies were heavily weighted with severe or chronic cases, there being a direct relationship between severity and concordance rate (Shields, 1966, discusses this topic fully).

Heston (1966) has compared the incidence of schizophrenia in two groups of children adopted in early life. Members of the first group of 47 children were born to schizophrenic mothers between 1915 and 1945 and of these five became schizophrenic; in the second group of children born to non-schizophrenic mothers none became schizophrenic.

Kety et al. (1968) report a study in which the biological families of children adopted in early life were compared in a series where half the children subsequently had developed

schizophrenia and half had not. In the former group schizophrenia was found significantly more often among members of the biological family.

Both these studies argue in favour of a genetic factor in schizophrenia.

To summarise the present position, it does not seem unreasonable to agree with the conclusion of Rosental (1968), who wrote in summerising the papers presented at a conference devoted entirely to the transmission of schizophrenia (and which was attended by geneticists and environmentalists),

"the case for heredity has held up convincingly."

The studies of metabolic transformation to be presented in the present thesis (in particular of methylation and of demethylation) are developed from the concept of schizophrenia as a disease which is genetically determined to a greater or lesser extent but these studies do not depend entirely on such a concept nor would a difference between the activities of enzymes in schizophrenics and non-schizophrenics amount to verification of a genetic hypothesis for schizophrenia. For any such difference to be designated as due to genetic variability, family studies would need to be presented.

(ii) <u>The mode of inheritance of schizophrenia, with a note on a polygenic hypothesis</u>. The present position has been summarised by

Slater (1967):

"The data at present available can readily be reconciled with more than one genetic hypothesis."

Slater himself has favoured the existence of a 'dominant' gene with incomplete penetrance; Kallmann favoured the operation of a 'recessive' gene. Other theories have suggested the operation of two genes simultaneously while yet others have suggested that a gene with dominant effect may operate to produce a mild form of the illness, while a different gene with a recessive effect may operate (in the homozygote) to bring about a more severe form of illness.

Gottesman and Shields (1967) argue in favour of polygenic determination for a large proportion of cases of schizophrenia. Polygenic inheritance would certainly explain what has been known and agreed upon for a long time, that classical Mendelian ratios are not found among the relatives of schizophrenic probands. It would also obviate the need to find heterozygote or other "advantage" which is implied by the view that schizophrenia is a genetic polymorphism (v.i.). Schizophrenia, on polygenic theory, would be regarded as "part of the genetic load, the price paid for conserving genetic diversity " (Gottesmann & Shields, 1967). According to this theory, genetic loading plus environmental loading could summate, leading to the crossing of a "threshold" so that clinical symptoms would result.

Known and as yet unknown polymorphic systems could, on such a theory, still remain relevant to the problem of schizophrenia just as blood group and secretor status remain relevant to the problem of peptic ulcer without contributing a great deal to the variance (i.e. to the genetic differences between those who have peptic ulcers and those who do not). Furthermore, rare recessive abnormalities (e.g. homocystinuria) could make a contribution to the total genetic variance between schizophrenics and non-schizophrenics of a significant, though minor, nature. This point can perhaps be illustrated best by a numerical example. Assume homocystinuria to occur in 1:100,000 of the population. Using the Hardy-Weinberg equation this would give the approximate frequencies for the abnormal gene of 0.003 and for the individual heterozygote for this gene of 0.006. Of a population of one million, 6,000 would be homocystinuria heterozygotes. If one were to postulate that these individuals are, by reason of the specified genotype, ten times as likely to develop a schizophrenic illness than the 0.9% morbidity risk for schizophrenia, then 9% or 540 such individuals in a population of one million would be homocystinuria heterozygote schizophrenics out of 9,000 who developed symptoms of schizophrenia during their lifetime, i.e. 6%. This would make a significant but minor contribution to the genetic loading in schizophrenia. The individual contribution of such effects to the

total genetic load in schizophrenia may be small : yet so large is the number of possible abnormalities that the total contribution may be considerable.

A polygenic theory draws attention to the need to study the activities of single enzymes in large rather than in small groups of subjects. If biochemical findings suggest that a certain enzyme is worthy of study, then, on a polygenic theory, the difference between schizophrenic and non-schizophrenic subjects will not be of an entirely clear cut nature and may be expected to emerge (if at all) only on statistical treatment of the data.

(iii) Schizophrenia and genetic polymorphism. Ford (1940) defined polymprphism as the occurrence in the same habitat of two or more discontinuous forms of the same species in such proportions that the rarest of them cannot be maintained by mutation.

The maintenance in such a situation is brought about by a gene conferring sometimes favourable, sometimes unfavourable characteristics on the various forms possessing it. The best known example of this in homo sapiens is the "sickle cell" gene, a single dose of which confers relative reistance to falciparum malaria while a double dose produces a haemolytic anaemia which carries a very high mortality before the reproductive age is reached. This situation

has been in balance because gene loss from sickle cell anaemia in the homozygote has been counterbalanced by gene preservation in the heterozygote due to lower mortality from malaria.

Huxley et al. (1964) have summarised evidence which would support the concept of schizophrenia as constituting an example of genetic polymorphism. As far as biological advantage is concerned, they cited evidence (much of which is methodologically suspect) purporting to show that schizophrenics are relatively resistant to surgical and wound shock, to visceral perforation, to histamine, to insulin, to thyroxine, to pain, to many allergies and "probably to many infections." If schizophrenia were due to the action of a single dominant gene with low penetrance, these advantages would raise the biological fitness of the non-schizophrenic carriers above that of non-carriers. This would be balanced against the lower reproductivity of those affected with schizophrenia and a genetic polymorphism could thus result. (It may be noted that Ford, 1965, states that "a unifactorial character must be polymorphic if found even in 1% of a considerable po;ulation," and Böök, 1960, considered the incidence of schizophrenia to be at least 1% in all racial and ethnic groups).

Erlonmeyer Kimling (1968) has recently produced data showing that the offspring of schizophrenics enjoy increased survival in the first year of life and that female offspring are more likely to

survive to the beginning of the reproductive period than the offspring of non-schizophrenics.

This needs confirmation, since earlier studies did not give evidence of a survival advantage in the children of schizophrenics. Furthermore, findings of this sort would achieve greater significance if it could also be shown that the children of non-schizophrenic first degree relatives of schizophrenics also had an increased chance of survival.

Moran has suggested (1965a) that the 'advantage' in schizophrenia might lie in a different mechanism from anything mentioned so far. Thus there is a tendency for schizophrenics to move down the social scale, and this may mean that they, or their offspring, adopt the increased fertility of the class to which they descend. Since, however, an inverse relationship between fertility and social class is not a general rule in all cultures, such an explanation of the stability of the polymorphism (if such there be) would not be entirely satisfactory. Moran (1965b) has also suggested that a biological advantage might lie in an increased probability of fertilisation resulting in productive pregnancy, similar to the effect by which ABO incompatability protects against Rh incompatability. Both the effects suggested by Moran would have to be shown to operate in the families of schizophrenics (since the schizophrenics themselves are manifestly less fertile than normal).

The acceptance of schizophrenia as an example of a genetic polymorphism cannot be regarded as by any means established for the evidence is incomplete and contradictory, as Baldessarini and Snyder (1965) have indicated.

It is worth pointing out that any advantage that a non-schizophrenic trait carrier might possess in the way of increased resistance to infectious disease would be more easily understood if the result of any gene fault were not limited to the brain alone. Thus, increased resistance to infection would mean more than just an increased resistance of that part of the brain which was affected by the schizophrenic process : it would imply a much more general increase in resistance. Increased resistance of a part of the brain to toxins could, nonetheless, exert a profound and widespread metabolic effect, if that part of the brain included the hypothalmus, and if effects were mediated via the pituitary-adrenal axis.

A metabolic abnormality lying wholly or partially outside the brain itself which confers some biological advantage but also favours the occurrence of a schizophrenic illness could satisfactorily fulfil the conditions which the concept of schizophrenia as constituting and example of genetic polymorphism would require. Such a concept of schizophrenia is not at variance with the principles of biochemical genetics nor with what is known of other

psychiatric disorders. If one wished to hypothesize that schizophrenia might be a disorder of brain function secondary to an enzyme abnormality outside the nervous system, one could instance acute intermittent porphyria as an example of a condition in which profound psychic disturbances can thus result (in that instance the enzyme defect is in the liver). Alternatively one could point to the possibility that a variant brain enzyme might be complemented by a similar variant in some other organ or organs (just as the enzymes responsible for the acetylation polymorphism occur (polymorphically) both in liver and jejunum; Jenne, 1963). It might be not on the brain variant but on the variant in the other organ or organs that the biological advantage depended.

To summarise, the concept of schizophrenia as an example of genetic polymorphism although not substantiated appears to be feasible and is worth further consideration. The situation of a primary metabolic abnormality in schizophrenia to some extent outside the central nervous system is consistent with such a concept and not inconsistent with present-day knowledge of biochemistry and biochemical genetics nor would such a situation be unprecedented from the psychiatric point of view.

EXPERIMENTAL SECTION

Sections 1 and 2 : STUDIES OF O-DEMETHYLATION.

The studies are divided into two sections. In the first the results of the use of in vitro methods is given. In the second an account of the reasons for abandoning this approach in favour of a pharmacogenetic one introduces a section concerned with the 4-O-demethylation in vivo of the substance methacetin, in control and schizophrenic subjects.

Section 3 : METHYLATION IN VIVO.

This comprises a comparative study in control and schizophrenic subjects of the metabolism of 3:4 dihydroxybenzoic acid (protocatechuic acid) with special reference to 3-O, 4-O and 3,4 di-O methylation.

EXPERIMENTAL SECTION.

Introduction to Sections 1 and 2.

Thus far evidence has been presented concerning an 'abnormal methylation hypothesis' in schizophrenia and this has subsequently been shown to be consistent with evidence indicating that schizophrenia is a condition to some extent genetically determined. The concept of schizophrenia as constituting an example of genetic polymorphism has been considered; the present evidence supporting such a concept is inconclusive.

From this general position the experimental investigation of specific hypotheses will now be considered.

The Demethylation Hypothesis To Be Tested.

This may be stated as follows:- that in some or all of those conditions described under the title schizophrenia there is a deficiency in the demethylating mechanism which allows methylated compounds to accumulate.

A review of data relating directly or indirectly to the demethylation hypothesis.

1. General considerations.

The brain must necessarily have the difficulty in dealing with circulating methylated compounds which is imposed by the fact that it appears to have no demethylating enzymes of its own.

O-demethylating enzymes appear to be located in liver microsomes, and have not been described elsewhere.

The immediate objection which might be made to the hypothesis that patients with liver dysfunction do not show the clinical syndrome of schizophrenia is met by the fact that the liver is an important source of methylating enzymes. Thus, Axelrod and Tomchick (1958) studied the methylation by catechol O-methyl transferase (C.O.M.T.) of l-epinephrine in a number of different organs from rats and found liver to be over three times as active per 100 G. of organ than any other organ studied (kidney, spleen, small intestine, lung, brain, heart muscle and skeletal muscle). Of these, kidney had 30% of the activity of liver; next most active was spleen (7%). Since the weight of liver is much greater than that of kidney, the liver (in the rat) must be regarded as being the major source for this enzyme.

Patients with liver dysfunction do occasionally show symptoms of a schizophreniform type (Read et al., 1967), paranoid schizophrenia being the form of schizophrenia appearing most commonly and one could hypothesize that failure of the demethylating system (which is unique to the liver) contributes, in these patients, to the psychosis. As with many hypotheses, however, plausible alternative theories can readily be constructed.

Hoffer (1967) has claimed benefit to occur in some cases of

acute schizophrenia from nicotinamide therapy. Nicotinamide is a cofactor for the demethylating enzyme and that benefit may occur when massive doses of a cofactor for a malfunctioning enzyme are administered has been shown in another connection. Thus Harrow et al. (1969) have shown that some cases of methylmalonic aciduria (a condition leading to failure to thrive, variable mental retardation, ketoacidosis and frequently to death in early life) respond to massive doses of vitamin B 12; the cobamide form of this vitamin is coenzyme for the enzyme deficient in methylmalonic aciduria, methylmalonyl - CoA carbonyl mutase.

Other 'abnormal methylation' hypotheses are based on the concept of abnormal methylated compounds being constructed in the body i.e. an increase or excess of methylation. Such a concept is consistent with modern thinking on biochemical genetics; for example in acute intermittent porphyria the abnormally rapid formation of porphyrins is consequent upon a defect in an operator gene. Generally, however, deficiency of an enzyme has been a commoner finding in the inborn errors of metabolism and the demethylation hypothesis would be consistent with this general situation.

The hypothesis would not be inconsistent with evidence that methyl donors (methionine and betaine) were deleterious in schizophrenia and that methionine antagonists were beneficial,

for any situation in which the formation of methylated compounds tended to increase or decrease would tend to accentuate or diminish (respectively) the effects of a demethylation impairment.

The demethylation hypothesis was formulated following the publication (originally in 1962) by Friedhoff and Van Winkle, of the claim that a substance behaving like dimethoxyphenylethylamine (D.M.P.E.) on paper chromatography was to be found in the urine of some schizophrenic subjects.

At the time that interest was focussed on D.M.P.E., an apparent weakness of the demethylation hypothesis was that the O-demethylation of this compound constituted only a minor pathway for its catabolism. However, the amounts of D.M.P.E. reported as being present were small ($< 100\,\mu G/24$ hrs), and an investigation into the possibility that the activities of the demethylating enzyme determined whether a very small amount of D.M.P.E. or none at all could be detected in the urine did not seem unreasonable. Furthermore, methylated non-amine precursors of D.M.P.E. (e.g. 3:4 dimethoxyphenylalanine) might also have been substrates for the O-demethylating enzyme which could, therefore, theoretically act at more than one level.

The rationale for the work presented here on demethylation is not dependent on there being D.M.P.E. in schizophrenic urine. What has been investigated is the activity of a demethylating

enzyme which is responsible for the O-demethylation of a large number of compounds, for Axelrod (1956) showed that the enzyme was active against substrates having a wide variety of side chain configurations. The enzyme may reasonably be presumed to enter into the catabolism of a large number of one ring psychomimetic compounds (such as those listed by Shulgin et al., 1969). Some of these will be poor substrates for monoamine oxidase because of their side chain configuration, and in these O-demethylation may attain more importance as a degradation process.

The in vitro study was carried out at a time when the presence of D.M.P.E. in the urine of schizophrenics was not disputed to the extent to which it is now and this work is presented here with reference to D.M.P.E. for that reason.

2. Psychopharmacological Considerations.

The cleavage that has been studied in vitro is the demethylation of para-methoxybenzaldehyde and para-methoxyacetanilide; in the in vivo studies the substrate used was para-methoxyacetanilide (Methacetin). Thus it has been 4-O-demethylation (i.e. demethylation in the para position) that has been studied (rather than demethylation in the 2-O or 3-O positions), throughout.

This is a deliberate choice. Smythies and Levy (1960) showed that the replacement of the 4 methoxy group of mescaline by a hydroxy group eliminates activity so that the 4-O-demethylation

of mescaline is a detoxification process (in the sense that a less toxic product results). Incidentally it is a process which rabbit liver microsomes are able to carry out (Daly et al. 1962), but not one which is important in the metabolism of mescaline in man (Cheraskopous et al., 1966).

Shulgin et al. (1969) in a study of the effects of one ring psychotomimetic compounds (chiefly phenylethylamines and phenylisopropylamines) on human subjects found that the addition of a para-methoxy group tended to increase the activity of a compound (i.e. tended to lessen the dose required to induce an effect equal to that of a given dose of mescaline). Furthermore, Smythies et al. (1967b) showed in a study of all possible phenylethylamines (using a Skinner box technique) that a para-methoxy group was necessary(but by no means sufficient) to bring about a mescaline-like effect in the rat. Michaux and Verly (1963) reached a similar conclusion. Smythies et al. (1967a) also tested a series of tri-, di- and mono-methoxy amphetamines (using similar techniques) and showed that the essential feature of the "hallucinogenic molecule" in this series of compounds was the para-methoxy group.

Barbeau et al. (1960) studied the pharmacological activity of a number of methoxyphenylethylamines on the mouse and found that a 4-methoxy group conferred on a compound the ability to produce akinesia. Thus 4-methoxy phenylethylamine was as active as D.M.P.E.

in this respect while 3-methoxyphenylethylamine was inactive. Ernst's (1962) findings are similar. He supposed "that in human patients phenomena of the hypokinetic rigid type are created by O-methylation of dopamine in the para-position...."

In a later paper (1965) Ernst reported the results of the testing of a larger number of substituted phenylethylamines; the hypokinetic rigid syndrome (H.R.S.) described by him consists of three components - active negativism, hypokinesia and catatonia; of nine compounds administered, the four with a para-methoxy group led to an H.R.S.; the five compounds having either an H or an OH in the para position all failed to show an H.R.S.

While it cannot be claimed that the states of hypokinetic rigidity such as Ernst produced in experimental animals by substituted 4-methoxyphenylethylamines are in any way equivalent to the catatonia of schizophrenia, the statement that a study of 4-O-demethylation has more relevance to the pharmacological findings than a study of 3-O-demethylation would appear to be a valid one.

It may be noted that the O-demethylating enzyme described by Axelrod (1956) O-demethylates more rapidly at the para (i.e. the 4) position. C.O.M.T. methylates preferentially in the meta (i.e. the 3) position. Thus the two enzymes do not exert direct antagonism.

EXPERIMENTAL SECTION 1

STUDIES OF O-DEMETHYLATION IN VITRO.

The basic reaction being studied was:-

$$ROCH_3 + \text{Oxygen} \rightarrow R.OH + H.CHO$$

(FORMALDEHYDE)

Enzyme activity was assayed by trapping the formaldehyde with semicarbazide and subsequently releasing it to produce a yellow dye, measurable spectrophotometrically.

The standard enzyme experiment

1. Materials

Tissue : two sources of tissue samples were used.

a) Rabbit Liver. Young male rabbits, starved for 24 hours, were killed by stunning, and the livers immediately removed and used at once, being kept throughout subsequent manipulations at 0 - 3°C.

b) Human Liver. Liver was collected from subjects on whom post-mortem was possible within three hours of death. It was placed immediately into a waxed container and kept thereafter at -20°C in a deep freeze until used. A note was made of the interval of time between death and post-mortem, and of all medication received by the patient in the period of two months before death.

Biopsy material taken during laparotomy for non-hepatic conditions proved unsuitable because the technique used here required more material than could safely be obtained at biopsy (a technique used by Kuntzman et al., 1966, using tagged substrate gets over this difficulty).

Fractionation of tissue homogenates.

A known weight of liver was homogenized with twice the weight of 0.05 M phosphate buffer, this containing KH_2PO_4 and K_2HPO_4 and having a final pH of 7.4. An Ultra Turrax homogenizer was used for this purpose, using 6 x 10 second bursts over a period of two minutes, this procedure keeping the temperature of the homogenate (which was within a boiling tube resting in an ice bath) below 3°C. The homogenate thus produced was divided up into conveniently sized equal portions and centrifuged at 9,000 G, in order to separate off unbroken cells, nuclei and mitochondria. The supernatant (microsomes and soluble fraction) was drawn off with a broken-tipped pipette in such a way as not to disturb the deposit resulting from centrifugation; all supernatants were pooled and kept at 0°C until required for use. Experiments were planned so that the period between completion of preparation of the homogenates and their use was reduced to a minimum - usually less than ten minutes.

This method of preparing homogenates is that described by Axelrod (1956), and cofactors were added in amounts proportional to those used by him.

Cofactors and other additions.

Three cofactors were used in these experiments -

T.P.N., nicotinamide and $MgCl_2$.

Amounts used were as follows:-

1. Microsomes and soluble fraction from approximately 2 G. rabbit liver or up to 4 G. human liver.
2. 16 μmol substrate.
3. 2 μmol T.P.N.
4. 50 μmol $MgCl_2$.
5. 100 μmol nicotinamide.
6. 400 μmol semicarbazide (neutralised).
7. 1.6 ml. 0.5 M KH_2PO_4/K_2HPO_4 buffer, pH 7.4.
8. Water to a final volume of 24 ml.

The amounts of T.P.N., $MgCl_2$ and nicotinamide were added in the form of appropriate amounts of solutions of these substances in water. Semicarbazide (used to trap any formaldehyde formed) was made up as a solution of the hydrochloride, brought to pH 7 with N.KOH and made up to a final exact volume such that 2ml. contained 400 μmol of semicarbazide.

2. Methods.

Incubation was carried out in stoppered flasks inserted into a water bath maintained thermostatically at 37°C. The flasks contained much more air than enzyme mixture; this air provided the supply of oxygen which was required for the reaction to proceed.

After incubation for 2 - 3 hours, 12 ml. aliquots of flask contents were removed for formaldehyde estimation and the

enzymatic reaction terminated by the addition of 4 ml. 20% aqueous zinc sulphate and 4 ml. of a saturated solution of barium hydroxide.

The resulting fluid, which contained a thick white precipitate, was centrifuged at 2,000 G. for ten minutes and the supernatant pipetted off and filtered through Whatman No.1 filter paper. 7 ml. of the resulting filtrate was used in the subsequent analysis, this providing enough solution for six estimates of formaldehyde content as follows (all figures being in ml.):-

Tube number	1	2	3	4	5	6
7 ml. filtrate	2	2	1	1	0.5	0.5
Water	3	3	4	4	4.5	4.5
Modified Nash's reagent (v.i.)	2	2	2	2	2	2
	7	7	7	7	7	7

Formaldehyde was measured by means of the modification that Cochin and Axelrod (1959) used of a method described by Nash (1953). The reagent consisted of 150 G. Analar ammonium acetate and 2 ml. acetyl acetone diluted to a final volume of 500 ml. with distilled water.

The six tubes each containing a total of 7 ml. were heated in a stirred water bath at 60°C for 30 minutes and the yellow dye thus formed was determined immediately in a Unicam 600 spectrophotometer set at 415 mμ.

An evaluation of the technique used for measuring formaldehyde, using the parallel lines experiment.

It was clearly necessary to demonstrate that nothing in the enzyme preparation interfered with the determination of its formaldehyde content.

The following experiments showed that this was the case. They also provided a standard line for the Nash reagent reaction. Amounts used in the enzyme experiment were scaled up so as to provide sufficient material for the analysis.

Experiment:

Materials:

The liver was obtained at post-mortem from a patient aged 60, who died from subarachnoid haemorrhage, acute bronchitis and haemorrhagic cystitis, in whom the liver was macroscopically normal. Medication in the days before death included 5 cc. I.M. Paraldehyde three days before death and 2 mega units of penicillin and 2 G. of streptomycin daily for two days before death. The liver had been in -20°C cold storage before use for six months.

Methods:

48 ml. was removed from at the end of an enzyme experiment (v.s.), and 16 ml. $ZnSO_4$ (20% aqueous solution) and 16 ml. $Ba(OH)_2$ added in turn. The resulting fluid was centrifuged at 2,000 G. in a bench centrifuge for ten minutes, and filtered

through Whatman No.1 filter paper. The resulting fluid was designated solution 1. Solution 2 consisted of a stock solution of formaldehyde, freshly made up and containing 10 μmol formaldehyde and 100 μmol neutralised semicarbazide per 100 ml., the latter being added to prevent formaldehyde losses due to evaporation.

Tubes were made up as follows (20 ml. stoppered exelos):-

Tube No.	1	2	3	4	5	6	7	8	9	10
Soln. 1	-	-	-	-	-	-	-	-	-	-
Soln. 2	0.5	0.5	1	1	2	2	3	3	4	4
Water	4.5	4.5	4	4	3	3	2	2	1	1
Nash reagent	2	2	2	2	2	2	2	2	2	2
Total	7	7	7	7	7	7	7	7	7	7

Tube No.	11	12	13	14	15	16	17	18	19	20
Soln. 1	1	1	1	1	1	1	1	1	1	1
Soln. 2	0.5	0.5	1	1	2	2	3	3	4	4
Water	3.5	3.5	3	3	2	2	1	1	0	0
Nash reagent	2	2	2	2	2	2	2	2	2	2
Total	7	7	7	7	7	7	7	7	7	7

Using the same solutions 1 and 2 further tubes were constituted as follows:-

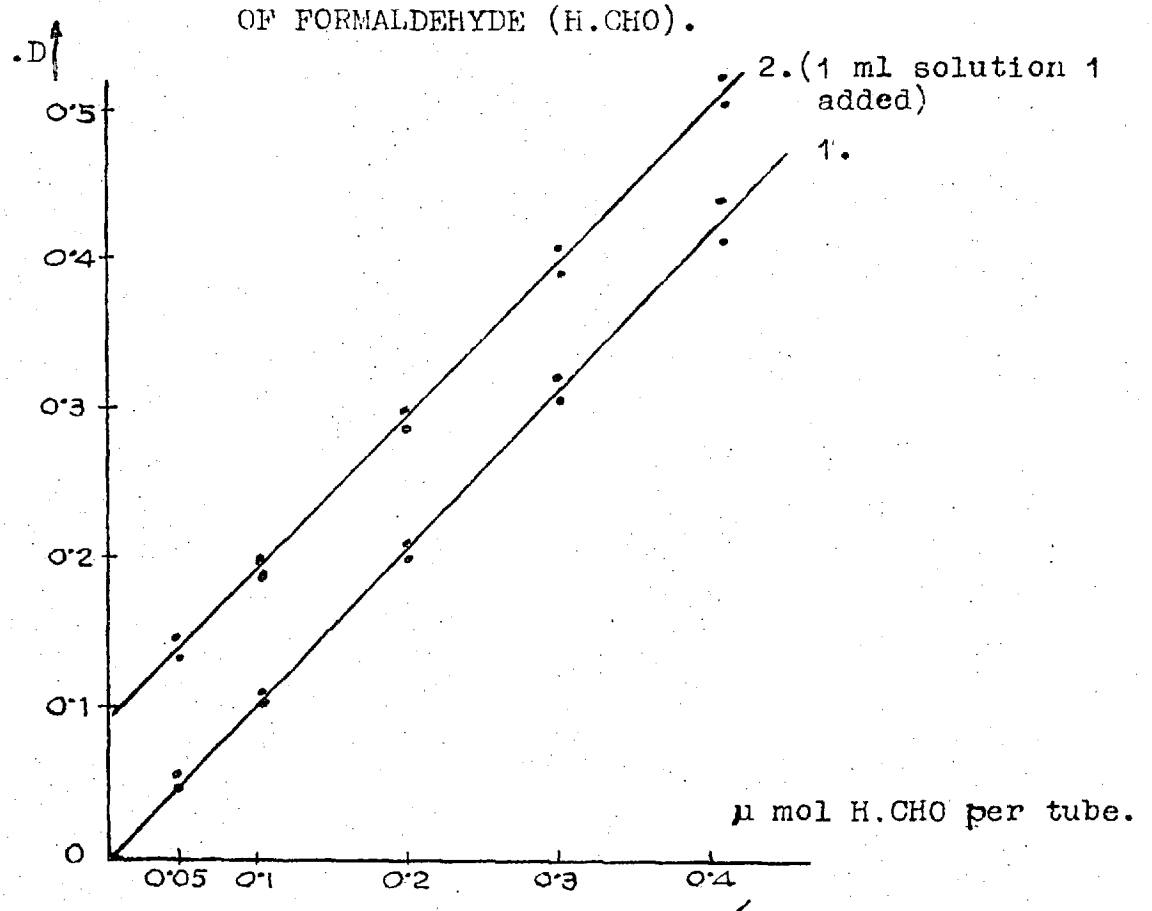
Tube No.	21	22	23	24	25	26	27	28	29	30
Soln. 1	0.5	0.5	1	1	2	2	3	3	4	4
Soln. 2	-	-	-	-	-	-	-	-	-	-
Water	4.5	4.5	4	4	3	3	2	2	1	1
Nash Reagent	2	2	2	2	2	2	2	2	2	2
Total	7	7	7	7	7	7	7	7	7	7

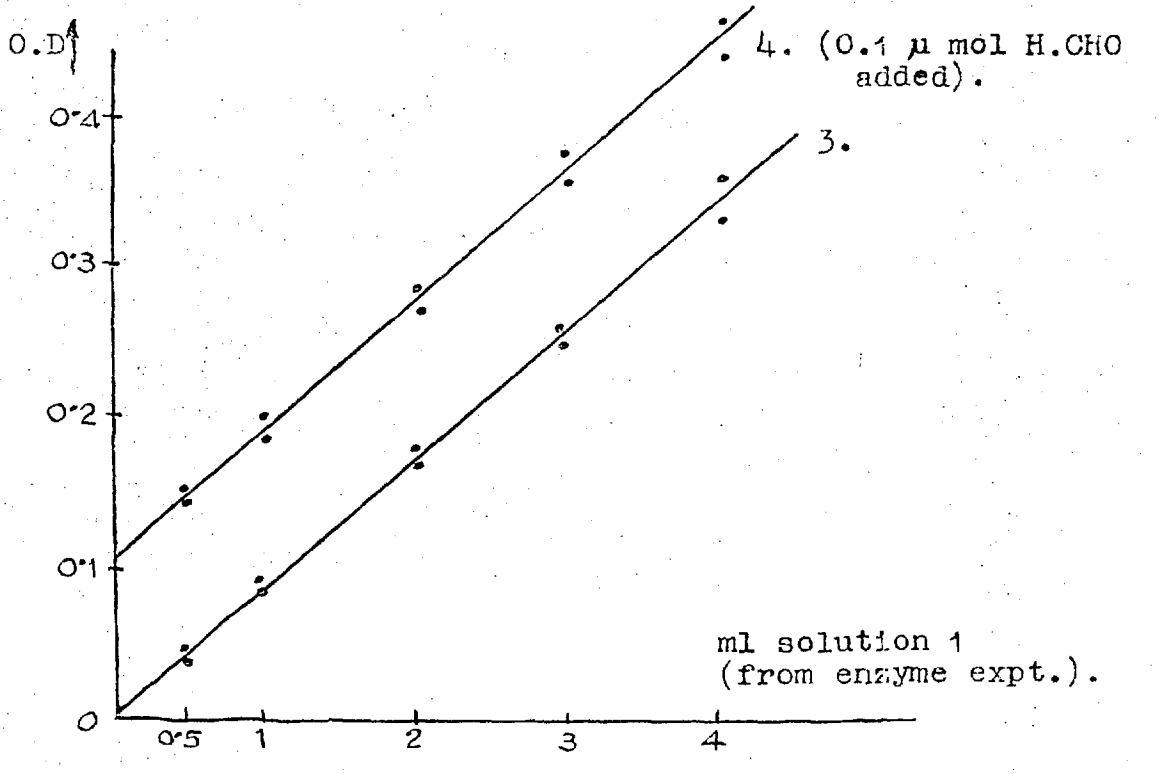
Tube No.	31	32	33	34	35	36	37	38	39	40
Soln. 1	0.5	0.5	1	1	2	2	3	3	4	4
Soln. 2	1	1	1	1	1	1	1	1	1	1
Water	3.5	3.5	3	3	2	2	1	1	0	0
Nash Reagent	2	2	2	2	2	2	2	2	2	2
Total	7	7	7	7	7	7	7	7	7	7

After heating in the water bath at 60°C for 30 minutes, the resulting colour was read at 415 mμ in a Unicam 600 spectrophotometer.

Unicam readings corrected for cell error were as follows:-

Figure 5. PARALLEL LINES EXPERIMENT FOR ESTIMATION OF FORMALDEHYDE (H.CHO).





Results

1. 0.049	6. 0.208	11. 0.137	16. 0.288
2. 0.053	7. 0.310	12. 0.145	17. 0.406
3. 0.101	8. 0.318	13. 0.165	18. 0.390
4. 0.105	9. 0.437	14. 0.194	19.0.515
5. 0.204	10. 0.409	15. 0.301	20.0.498
21. 0.041	26. 0.179	31. 0.152	36. 0.271
22. 0.044	27. 0.260	32. 0.147	37. 0.363
23. 0.085	28. 0.249	33. 0.186	38. 0.377
24. 0.087	29. 0.356	34. 0.196	39. 0.440
25. 0.172	30. 0.334	35. 0.280	40. 0.457

From these 40 readings, four lines were constructed.

Line 1 (tubes 1 - 10), Line 2 (tubes 11 - 20), Line 3 (tubes 21 - 30) and Line 4 (tubes 31 - 40) as shown in Fig. 5.

If all lines have the form $y = a + bx$, where x and y are as shown in Fig. 5, b is the slope of each line, and a the intercept on the y axis, then the data obtained yield the following values.

	b	b/Se (b)	p for $\frac{b}{Se(b)}$	a	a/Se(a)	p for $\frac{a}{Se(a)}$
Line 1	1.062	78	0.001	-0.0036	-0.36	N.S.
Line 2	1.045	58	0.001	0.0865	14.6	0.001
Line 3	0.0859	49.6	0.001	0.00031	0.073	N.S.
Line 4	0.0864	95	0.001	0.105	45	0.001

Se(a); Se(b) = Standard error for a, b respectively.

p is the probability coefficient.

All values of p for the slopes, b_n, are highly significant.

The t test for the differences between the values of b for Lines 1 and 2 gives a value for t of 0.6, and this is not significant. There is a significant intercept (a) for line 2, with a value of 0.0865 units (of y).

The t test for the difference between the two values of b for lines 3 and 4 gives a value for t of 0.256, and this is not significant.

There is a significant intercept (a) for line 4 with a value of 0.105.

Further considerations (see Fig. 5).

The intercept (a), for line 4 represents the value of y due to the addition of 1 ml. of solution 2, i.e. of 0.1 μmol of formaldehyde. Ignoring the tiny intercept, the slope of line 1 is given by the expression $\frac{y}{x}$, i.e. $b_1 = \frac{\text{Unicam reading } (=y)}{\mu\text{mol formaldehyde per 7 ml. final solution.}}$

The value of y when the denominator (x) is 0.1 is $b_1 \times 0.1 = 0.1062$, i.e. 0.1 μmol formaldehyde/ 7 ml. yields a Unicam value of 0.1062 from line 1, and 0.105 from line 3 and 4, a highly satisfactory agreement.

Similarly, the intercept for line 2 (0.0865) represents the result of the addition of 1 ml. of solution 1.

Now the slope of line 3 is given by the expression

$b_3 = \frac{\text{Unicam reading}}{\text{ml. of solution analysed}}$, (ignoring the tiny intercept).

When ml. of solution analysed is made equal to 1, the Unicam reading is 0.0859. Both values (0.0865) and (0.0859) represent the optical density due to 1 ml. of solution 1. They clearly show very satisfactory agreement.

Conclusion.

This experiment shows that interference with the estimation of formaldehyde by substances included in the final solution (i.e. after zinc sulphate and barium hydroxide additions) is not, with the liver used here, taking place.

A note on the 't' test procedure applied to the "parallel lines" experiment and to 'in vitro' experiments generally.

The statistical nomenclature employed here and elsewhere is that used by Bailey (1959). The problem is to compare the slopes of two lines $y = a_1 + b_1x$ and $y = a_2 + b_2x$, b_1 and b_2 being the slopes. The assumption is made that the unknown residual variance is equal for the two lines. Bailey gives the following formula for Student's t

for the difference between slopes b_1 and b_2:

$$t = \frac{b_1 - b_2}{s\sqrt{\frac{1}{\Sigma_1(x-\bar{x}_1)^2} + \frac{1}{\Sigma_2(x-\bar{x}_2)^2}}}$$

where $s^2 = \frac{(n_1 - 2)\, s_1^2 + (n_2 - 2)\, s_2^2}{n_1 + n_2 - 4}$

where the number of degrees of freedom is $n_1 + n_2 - 4$.

In the present experiments, $(x - \bar{x}_1)^2 = (x - \bar{x}_2)^2$ because values for x are fixed beforehand, y being the only independent variable. Also $n_1 = n_2$.

Hence $t = \frac{(b_1 - b_2).\ \sqrt{\Sigma(x - \bar{x})^2}}{\sqrt{s_1^2 + s_2^2}}$ with $2n - 4$ degrees of freedom.

Choice of substrate used in the assay of demethylation enzymic activity.

The original intention had been to study the demethylation of 3 : 4 dimethoxyphenylethylamine (D.M.P.E.) and this indeed was carried out, as described below. This substrate has certain disadvantages.

1. Under the experimental conditions used, liver monoamine oxidase will attack D.M.P.E. Therefore a monoamine oxidase inhibitor will be required and that this will exert inhibition on the demethylating enzyme is suggested by the work of Daly et al.(1962)

who noted that iproniazid, a monoamine oxidase inhibitor, inhibited the demethylation of mescaline.

2. Human liver as collected at post-mortem is of very sluggish activity when compared with rabbit liver. Furthermore, the demethylation of D.M.P.E. proceeds slowly relative to the demethylation of other compounds, as will be shown. Hence formaldehyde yields are inconveniently low when D.M.P.E. is the substrate and human liver the enzyme source.

Axelrod (1956) listed relative activity of the demethylating enzyme against a variety of substrates. Using the O-demethylation of p-methoxyacetanilide as representing 100% activity, mescaline (3:4:5:trimethoxyphenylethylamine) was demethylated at 13% of that rate; the percentage for p-methoxybenzaldehyde, on the other hand, was 155, and this substance was chosen as being convenient for study, as it was commercially available in pure form.

Axelrod described the existence of two ether cleaving systems having different responses to the enzyme inhibitor SKF 525A. (This is the diethylaminoethanol ester of diphenylpropylacetic acid). The system responsible for the cleavage of aromatic ethers containing a benzene ring is more resistant to this inhibitor than the system cleaving codeine and morphine. Axelrod showed that a 20-fold excess of codeine had no effect on the demethylation of p-ethoxyacetanilide whereas a 20-fold excess of p-methoxybenzylamine markedly inhibited cleavage. Use of this method of inhibition of cleavage (of one substrate by adding a 20-fold excess of a second) in experiments presently to

be described showed that D.M.P.E. and p-methoxyacetanilide (Methacetin) were cleaved by the same enzyme in human liver (v.i. : Experiment 4). Methacetin was used subsequently for in vivo demethylation studies.

Difficulties in the experimental evaluation of the demethylation hypothesis, in vitro.

1. Differences between human and rabbit liver.

Apart from the comparatively sluggish activity obtained from human liver compared with rabbit liver, certain other differences were detected.

a. If an homogenate of rabbit liver was added to a flask containing not only cofactors, substrate and semicarbizide, but also 20% zinc sulphate so that the enzyme was immediately inactivated, no formaldehyde was detectable in the resulting fluid using the usual method of estimation with Nash's reagent. With human liver, however, small amounts of formaldehyde were apparently detectable (see Expt. 1, p 89), as shown by the observation that the yellow colour produced with Nash's reagent had a spectrum identical with that produced by authentic formaldehyde, with a peak at 415 mμ. By taking portions of liver just beneath the capsule and at much deeper levels, it was shown that the former contained more formaldehyde. This suggested surface contamination of the liver with formaldehyde within the post-mortem room as a likely and feasible explanation.

b. Rabbit liver incubated with cofactors, semicarbazide and buffer but no substrate yielded no formaldehyde. Human liver similarly treated yielded formaldehyde far in excess of that extractable using the preincubation inactivation described above, (see Expt. 1, p 95).

The explanation of this phenomenon is not clear, but an original theory that it might be due to detoxification of drugs within the liver at the time of death seems, on further consideration, unlikely. While it is true that such drugs as morphine and codeine (which are often administered in terminal illness) have methyl groups which can be cleaved by demethylating enzymes and that these drugs might be present in the liver at death, the amount present in an enzyme experiment in which only 0.1% of the whole liver was used would be much too small to account for the yield of formaldehyde obtained.

Thus, if the total body content of codeine were 50 mgm. at death, and 5% of this were in the liver, (this is the percentage for liver given by Latham and Elliott(1951) who studied the tissue distribution of C^{14} codeine in rats), 0.1% of the liver would contain 2.5 μG or 0.01 μmol. of codeine. The likely yield of formaldehyde from such a source during an enzyme experiment would be much less than this and insignificantly small.

c. A further difficulty was encountered in cases of sudden death

in humans, namely that fat caused troublesome opalescence so that formaldehyde estimation was interfered with. The use of homogenates prepared from unstarved rabbits was subject to the same difficulties, but in this case the situation could be rectified by experimenting with liver from starved animals.

It will thus be apparent that there are certain difficulties in applying a technique which worked eminently well with rabbit liver to human post-mortem material. The intention was that a number of livers from non-schizophrenic individuals should be compared with regard to their activity in demethylating codeine and their activity in demethylating p-methoxybenzaldehyde, each substance representing a different system of ether-cleaving enzymes. For each liver a ratio of the activity of these two systems/could have been determined and such ratios would be likely to be more or less independant of post-mortem change. Ratios for non-schizophrenic subjects could then have been compared with ratios for schizophrenics.

2. <u>The difficulties experienced in obtaining liver from schizophrenics.</u>

In practice, the difficulties already mentioned were slight compared with the difficulty experienced in obtaining post-mortem material collected within a few hours of death from schizophrenics. In spite of installing a portable deep freeze unit in a mental hospital mortuary, and frequent reminders to all concerned -

sick-ward nursing staff, post-mortem attendants and pathologists, no material was forthcoming; one reason appeared to be the long delay between death and post-mortem, this depending partly on the fact that the pathologist was not based on the mental hospital at which the schizophrenic had died. Attempts at a number of different hospitals met with equal lack of success.

Experiments carried out with post-mortem material established certain facts on which subsequent in vivo procedures were based, and are thus relevant to subsequent work.

SUMMARY OF IN VITRO EXPERIMENTS USING HUMAN TISSUES.

1. The demethylation of p-methoxybenzaldehyde and D.M.P.E. using appropriate controls, by human liver.
2. The dependence of human liver demethylating enzyme on various cofactors.
3. The products of demethylation of D.M.P.E.
4. Inhibition of the demethylation of p-methoxyacetanilide (Methacetin) by D.M.P.E.

1. The demethylation of p-methoxybenzaldehyde and D.M.P.E. using appropriate controls, by human liver.

The purpose of this experiment is to show the ability of human liver homogenate to demethylate p-methoxybenzaldehyde (P.M.B.A.), and D.M.P.E., and also to estimate the recovery of formaldehyde. And to compare the formaldehyde yielded by these

substrates with that yielded in the absence of substrate, and with that yielded by inactivation of the enzyme at the outset of the experiment.

Materials: The liver used was the same one used for the parallel lines experiment.

Methods:

12 G. of the liver was homogenized with 24 cc. 0.05 M. Sorensen's Buffer (pH 7.4). 20 ml. of liver preparation (microsomes and soluble fraction) were prepared as described above; it was calculated that each 4 ml. of this preparation corresponded to approximately 1.6 G. of liver.

The constituents of the flasks used were as follows, all figures being in ml. :-

	(1)	(2)	(3)	(4)	(5)
Liver prepn.	4	4	4	4	4
Nicotinamide 50 μmol.) $MgCl_2$ 25 μmol.)	2	2	2	2	2
T.P.N. 1 μmol.	0.2	0.2	0.2	0.2	0.2
Neutralized semicarb. 200 μmol.	0.5	0.5	0.5	0.5	0.5
0.5 mol. Sorensen's Buffer pH 7.4	0.8	0.8	0.8	0.8	0.8
8 μmol pM.B.A.	1	-	-	1	-
8 μmol D.M.P.E.	-	1	-	-	-
1 μmol formaldehyde	-	-	1	-	-
Water	3.5	3.5	3.5	3.5	4.5
20% Aq. $ZnSO_4$	-	-	-	4	-
Saturated $Ba(OH)_2$	-	-	-	4	-
	12	12	12	20	12

Note that a monoamine oxidase inhibitor was not used in this experiment.

The flasks were incubated in a water bath, with shaking, at 37°C for two hours. At the end of this time additions of 4 ml. of

20% Aq. $ZnSO_4$ and 4 ml. saturated $Ba(OH)_2$ were made to all flasks except flask 4.

The resulting suspensions were centrifuged at 2,000 G. for ten minutes, filtered through Whatman No.1 filter paper and for each flask a series of tubes was made up:-

	A	B	C	D	E	F
Filtrate	0.5	0.5	1	1	2	2
Water	4.5	4.5	4	4	3	3
Modified Nash's Reagent	2	2	2	2	2	2
	7	7	7	7	7	7

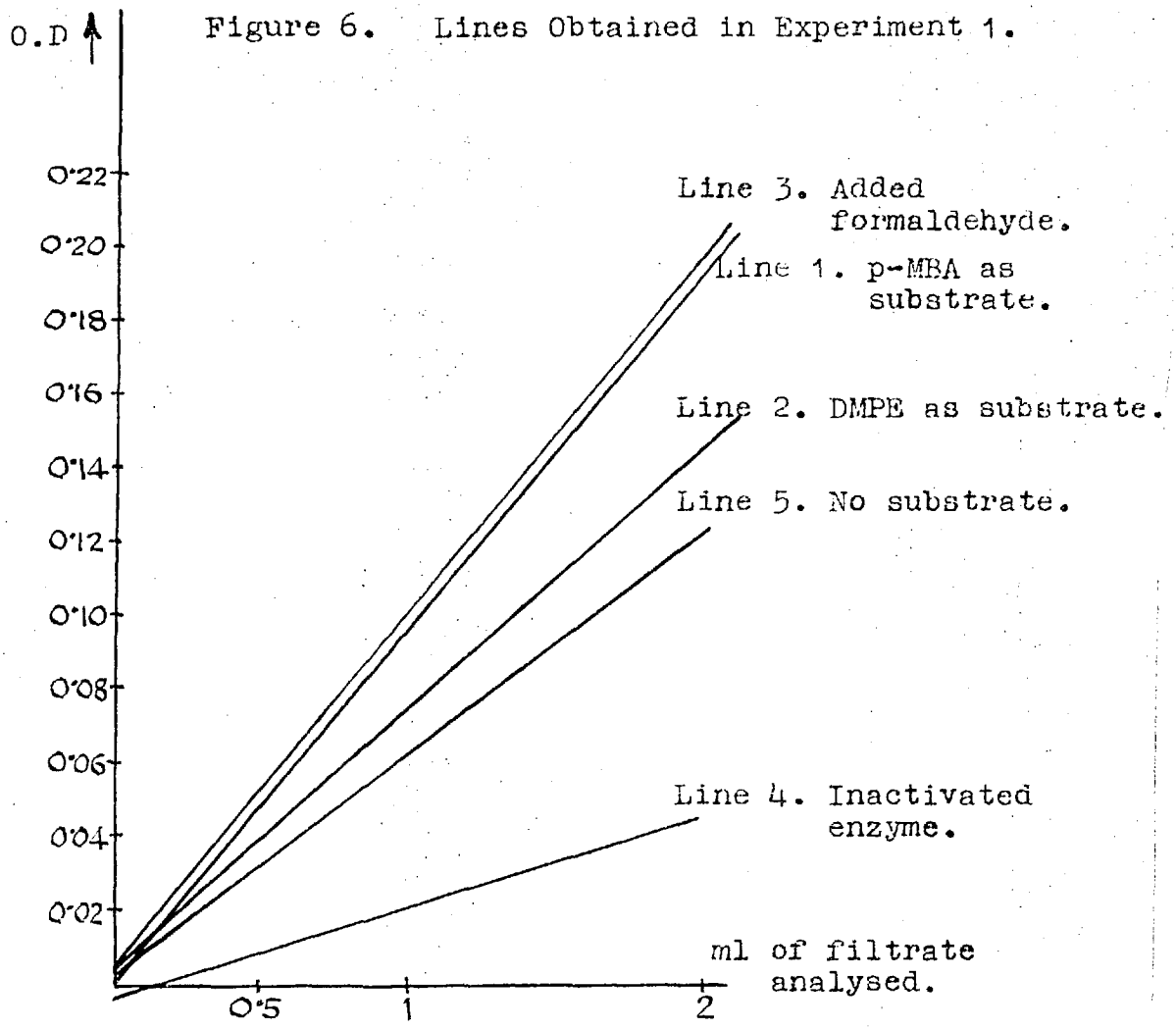
Colour was developed in the usual way and read at 415 mμ in a Unicam 600 spectrophotometer.

Results:

The following values (corrected for cell error) were obtained:-

	A	B	C	D	E	F
Flask No. 1	0.052	0.046	0.096	0.096	0.196	0.201
2	0.042	0.036	0.073	0.077	0.149	0.147
3	0.048	0.046	0.107	0.105	0.196	0.201
4	0.011	0.007	0.022	0.015	0.043	0.046
5	0.027	0.029	0.057	0.060	0.123	0.120

Figure 6. Lines Obtained in Experiment 1.



Statistical Evaluation.

If the lines given by this data are represented by

$$y = a + bx$$

where y = optical density and x = mils. of filtrate analysed, the following values are obtained:-

	b	b/Se(b)	p for b/Se(b)	a	a/Se(a)	p for a/Se(a)
1	0.1001	54.1	<0.001	-0.0022	-0.89	N.S.
2	0.0727	37.5	<0.001	+0.0025	0.98	N.S.
3	0.0998	30.4	<0.001	+0.0008	0.183	N.S.
4	0.0240	11.1	<0.001	-0.004	-1.38	N.S.
5	0.0624	53.8	<0.001	-0.0025	-1.63	N.S.

These lines are shown on Fig. 6

(b = slope of line, a = intercept of line on y axis).

The value for Student's t (ignoring sign) between pairs of flasks (with 8 degrees of freedom), are given by the following matrix:-

	1	2	3	4	5
1	-				
2	9.8	-			
3	0.08	7.1	-		
4	26.7	11.0	19.2	-	
5	17.2	4.6	10.7	15.6	-

All values for t are significant with $p < 0.001$, except that between flask 1 and 3 which is not significant ($p > 0.1$)

Further results and conclusions.

A. Recovery of formaldehyde.

This can be derived from the results from flasks 3 and 5, the composition of which differed only in that flask 3 contained 1 μmol of formaldehyde. At the level of 1 cc. filtrate analysed (and neglecting the small intercepts), flask 3 gives an O.D. of 0.0998, and flask 5 an O.D. of 0.0624, a difference of 0.0374.

The intercept of 0.105 in standard line 4 (p 81) represents the effect of the addition of 0.1 μmol formaldehyde to each exelo tube.

From this, a difference of 0.0374 in O.D. represents an amount of formaldehyde equal to $0.0374 \times \frac{0.1}{0.105} = 0.0356$ μmol formaldehyde.

The original 20 ml. from which the 1 ml. was derived will, therefore, have contained $20 \times 0.356 = 0.712$ μmol, that is to say, recovery in this experiment is 71.2%. This value is only approximately true, since the 20 ml. contained a precipitate, and the volume and formaldehyde content of thiswere not estimated separately.

B. Formaldehyde production from p-methoxybenzaldehyde.

The fact that lines for flasks 1 and 3 are so similar, yields the information that the amount of formaldehyde yielded from the

8 μmol substrate in flask 1 is 1 μmol (the amount added, in the absence of any substrate to flask 3).

C. Formaldehyde production from D.M.P.E.

The difference in formaldehyde yielded between flasks 2 and 5 is given by the expression $\frac{20 \times (0.0727 - 0.0624)}{1.05} = 0.196 \mu mol.$ Correcting for losses by multiplication by $\frac{100}{71.2}$ gives a value for yield of 0.28 μmol.

From this it appears that compared with p-methoxybenzaldehyde, only about a quarter of the amount of D.M.P.E. is demethylated under the conditions of this experiment.

D. Formaldehyde production in the absence of substrate.

(This phenomenon was discussed in an earlier section; the present results illustrate it).

This is shown by the difference between flask 5 and flask 4. The reason for this is not clear; study of what is known about the metabolism of the drugs administered before death (penicillin, streptomycin and paraldehyde) do not suggest that these could be responsible, even if they were present in sufficient concentration. It could be hypothesized that early post-mortem autolysis provided substrate(s) on which the demethylating enzymes acted when cofactors were provided and incubation carried out and that this might account for the difference noted between post-mortem human liver and the fresh liver obtained for studies on rabbit liver.

2. <u>The dependence of human liver demethylating enzyme on various cofactors</u>.

The demethylating system described by Axelrod (1956) depended on various factors : T.P.N., $MgCl_2$ and nicotinamide. Using p-ethoxyacetanilide as substrate, Axelrod obtained the following yield of formaldehyde - Axelrod's figures have been converted into percentages for convenience.

Complete system	100%
T.P.N. omitted	26%
$MgCl_2$ omitted	95%
Nicotinamide omitted	60%

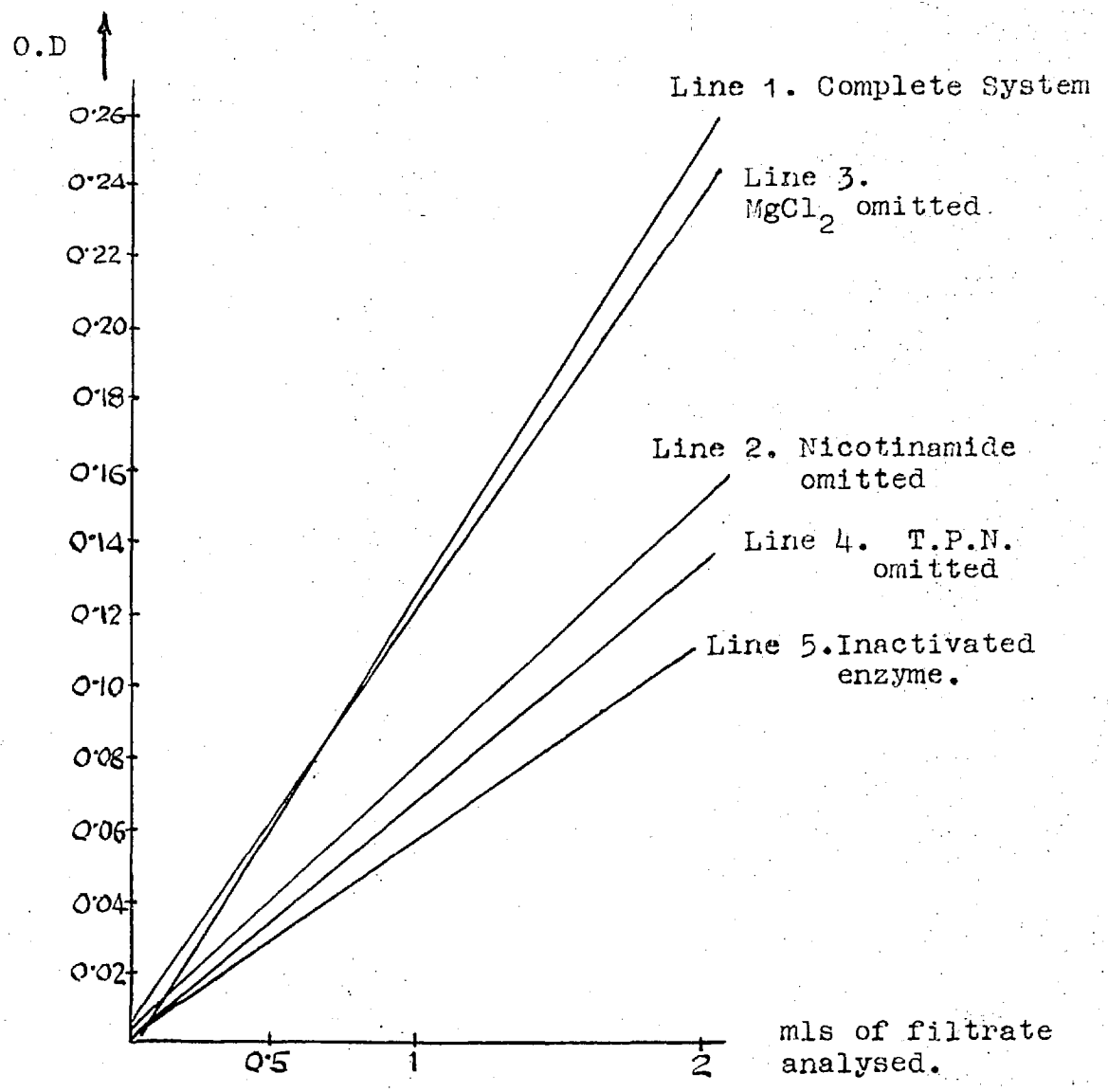
The object of the experiment to be described was to determine whether the human liver demethylating enzyme was also dependent on these cofactors.

Materials and Methods:

Using the same liver (with identical preparation) as for experiment 1, the following flasks were constituted, all figures representing ml. amounts:-

Flask Number	1	2	3	4	5
Liver preparation	4	4	4	4	4
Nicotinamide 50 μmol	1	-	1	1	1
$MgCl_2$ 25 μmol	1	1	-	1	1
T.P.N. 1 μmol	0.2	0.2	0.2	-	0.2
Neutralized semicarbazide	0.5	0.5	0.5	0.5	0.5
0.5 mol Sorensen's buffer pH 7.4	0.8	0.8	0.6	0.8	0.8
8 μmol p-methoxy-benzaldehyde	1	1	1	1	1
Water	3.5	4.5	4.5	3.7	3.5
20% Aq. Zn SO_4	-	-	-	-	4
Saturated $Ba(OH)_2$	-	-	-	-	4
TOTAL VOL.	12	12	12	12	20.
After incubation at 37^{0}C for 2 hrs. further additions were made:-					
Nicotinamide 50 μmol	-	1	-	-	-
$MgCl_2$ 25 μ mol	-	-	1	-	-
T.P.N. 1 μmol	-	-	-	0.2	-
Water	1	-	-	0.8	1
20% Aq. $ZnSO_4$	4	4	4	4	-
Saturated $Ba(OH)_2$	4	4	4	4	-
	21	21	21	21	21

Figure 7. THE EFFECT OF COFACTOR OMISSION ON HUMAN DEMETHYLATING ENZYME.



Line numbers correspond to those used for flasks.

Tubes were prepared in the usual way , and the amount of formaldehyde estimated in 2 ml., 1 ml., and 0.5 ml. of filtrate as described for experiment 1; 5 lines were thus constructed (see Fig. 7).

Results:

(Unicam readings at 415 mμ , corrected for cell error)

	0.5 cc. filtrate Duplicates		1 cc. filtrate Duplicates		2 cc. filtrate Duplicates	
Flask 1	0.065	0.059	0.126	0.122	0.247	0.263
2	0.039	0.036	0.076	0.079	0.150	0.150
3	0.058	0.058	0.121	0.112	0.235	0.247
4	0.037	0.032	0.064	0.069	0.142	0.131
5	0.028	0.025	0.060	0.057	0.107	0.117

Statistical Evaluation:

Using standard regression correlation statistics on this data with y = Unicam reading and x = mls. of filtrate analysed, and y = a + bx.

	b	a	$\frac{b}{Se(b)}$	p
Flask 1	0.129	-0.0035	31	p<0.001
2	0.0746	+0.0012	55.3	p<0.001
3	0.122	-0.0043	30.2	p<0.001
4	0.0683	-0.0005	21.4	p<0.001
5	0.0565	-0.0003	19.1	p<0.001

None of the values for a, the intercept, differs significantly from zero.

't' calculated from:		t	p (8 degrees of freedom).
Data from flasks	1 and 3	1.2	N.S.
" " "	1 and 2	12.4	<0.001
" " "	1 and 4	11.6	<0.001
" " "	4 and 5	2.63	<0.05
" " "	2 and 5	5.87	<0.01

The results are shown graphically in Fig. 7

Discussion and Conclusions.

It is evident that the omission of $MgCl_2$ had no significant effect on the formaldehyde yield. T.P.N. and nicotinamide omission both had a significant effect on yield, and on this evidence may be regarded as cofactors for human liver demethylating enzyme. If the yields are compared with base line i.e. inactivated enzyme, and referred to the figure of 100%, representing the complete system, the omission of nicotinamide reduced yield to 29% of that obtained when nicotinamide was included; similarly omission of T.P.N. reduced the yield to 17% of that obtained when it was included.

3. The identification of the products of demethylation of DMPE using human liver.

Introduction:

In this experiment the intention was to determine whether 3-hydroxy 4-methoxyphenylethylamine (3-OH 4-OCH_3. PEA) or

4-hydroxy 3-methoxyphenylethylamine (4-OH 3-OCH_3.PEA) were formed, or whether both were formed, when DMNE was demethylated. A monoamine oxidase inhibitor (phenelzine) was used to prevent the breakdown of DMNE by amine oxidase.

A method of separating the two possible mono-demethylated derivates of D.M.N.E. was established by Senoh et al. (1959) who were approaching the problem from the opposite direction, so to speak, in a study of the methylation of dopamine. Their special method (which has been used here) was developed because of failure to separate the two isomers on a number of solvent systems; this method involved azo-coupling of the amines while in solution (using diazotized sulphanilic acid) prior to chromatography. Their extraction procedure has also been used in the present experiment.

It was found by preliminary studies that semicarbazide interfered with this coupling process, and it was therefore omitted.

Materials:

The same liver was used for this experiemont as was used for experiments 1 and 2.

Methods:

The following were incubated together, using standard cofactor solutions. Only Flask 1 contained substrate (D.M.P.E.) All numbers in columns 1 and 2 represent volumes in ml.

FLASK NO.		1	2
1	Liver preparation	12.0	12.0
2	$MgCl_2$ 75 μmol Nicotinamide 150 μmol	6.0	6.0
3	T.P.N. 3 μmol	0.6	0.6
4	0.5 mol. Sorensen's phosphate buffer pH 7.4	2.4	2.4
5	32 μmol DM.P.E.	4.0	-
6	Phenelzine 3.6 μmol	1.0	1.0
7	Water	10.0	14.0
		36	36

This gives a final concentration of phenelzine of 10^{-4} mol. The flasks were incubated for three hours at 37°C. At the end of this time the reaction was terminated in both flasks by adding 5 ml. 30% trichloroacetic acid; 32 μmol of D.M.P.E. in 4 ml. water was added to Flask 2, and 4 ml. water to Flask 1. The precipitate was brought down in a centrifuge at 2,000 r.p.m. for ten minutes and the supernatant removed by pipette. The residue was washed twice with 10 cc. 0.1N hydrochloric acid, these washings being added to the supernatant.

The pooled supernatant and washings were adjusted to pH 10 with strong alkali and then shaken with 25 ml. of a 3 : 2 mixture of isoamylalcohol and toluene in order to extract the amines.

After separation, the upper (inorganic) phase was shaken again with 25 ml. of the organic solvent mixture. This was allowed to separate, and the organic phases containing the amines were pooled and shaken with 5 ml. 0.1 N HCl, for 15 minutes.

A sample of authentic 3-OH 4-OCH_3.PEA (the only one of the two isomers available commercially at that time) was taken separately through from this stage onwards, being made up as a solution of 500 µG in 5 ml. 0.1 N HCl.

4 ml. of the acid phase was removed and rendered neutral by the addition of 0.4 ml. N NaOH; to this were added-

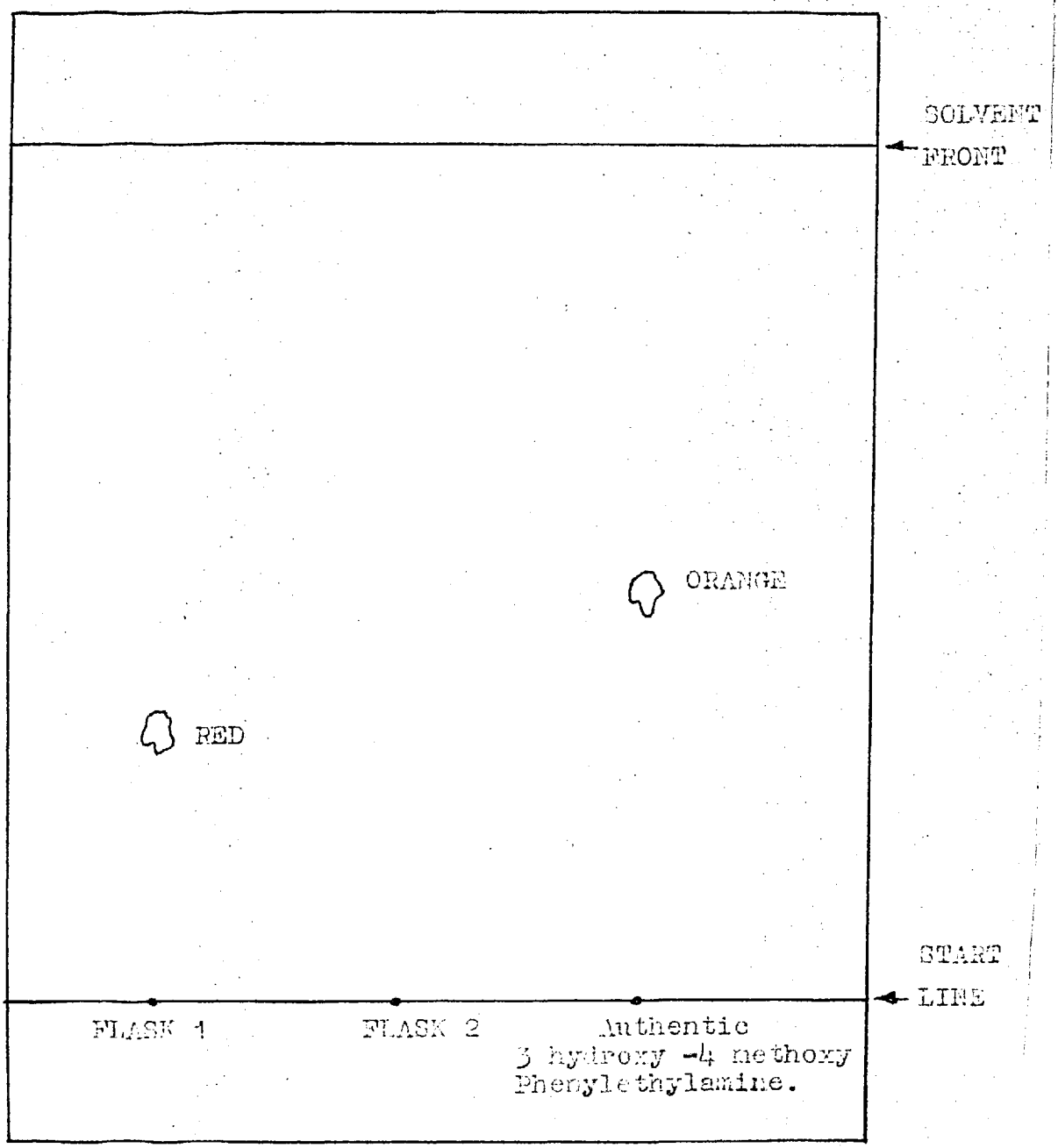
0.6 ml. of 10% NaOH and

0.4 ml. of freshly diazotized sulphanilic acid.

The latter was made up using the method of Ames and Mitchell (1952), in which 25 ml. 5% aqueous sodium nitrite is reacted with 5 ml. of stock sulphanilic acid reagent (sulphanilic acid 0.9 G; concentrated hydrochloric acid 9 ml; and water to 100 ml.), the reaction mixture being kept chilled.

The solution containing the azo-coupled amines was evaporated to dryness in vacuo. The residue was taken up in 0.2 ml. of methanol/water (equal parts), and 25 µL spots were placed on to Whatman No.1 paper which had previously been soaked in 2.5% aqueous Na_2CO_3 and dried. The chrometograms were developed (descending run) in methanol : benzene : butanol : water :: 2 : 1 : 1 : 1 for two hours and were then dried with a hot air fan.

Figure 8 The chromatogram obtained in experiment 3.



Solvent; Methanol : Benzene : Butanol : Water :: 2 : 1 : 1 : 1

Descending chromatography. Whatman No.1.

Results:

The area of the chromatogram derived from Flask 2 remained blank. The area of the chromatogram derived from Flask 1 showed a single red spot having an Rf of 0.35.

The area of the chromatogram derived from authentic 3-OH 4-OCH_3.PEA showed a single orange spot having an Rf of 0.50 (see Fig. 8).

Discussion:

The Rf values and colours obtained by Senoh et al. (1959) for authentic 3-OH 4-OCH_3.PEA and 4-OH 3-OCH_3.PEA (similarly chromatographed and stained) were 0.45, orange; and 0.31, rose red respectively. It would appear likely that the spot appearing on the chromatograms derived from Flask 1 resulted from 4-OH 3-OCH_3.PEA. Its presence only when D.M.P.E. was included in the original enzyme mixture suggested that it was a metabolite of this compound. The colour produced agreed with that found by Senoh et al. for authentic 4-OH 3-OCH_3.PEA and the Rf agreed fairly well with their figure.

Provided that the two amines under consideration extract to an equal extent under the conditions of extraction and provided that they couple with the azo-reagent to produce colours of equal intensity, then 4-O-demethylation of D.M.P.E. is much greater in amount than 3-O-demethylation (which, indeed, was not detected in

the present experiment).

This agrees with what Axelrod (1956) found in his studies with animal liver - that 4-O-demethylation takes place with greater facility than 3-O-demethylation. As mentioned elsewhere the product of demethylation, 4-OH 3-OCH_3.PCA has less pharmacological activity than D.M.P.E. itself.

Sargent et al. (1967) examined the demethylating activity in intact rats by injecting them with D.M.P.E. which had been ^{14}C tagged either on the 4-methoxy group or the 3-methoxy group; they found that expiration of $^{14}CO_2$ was about 15 times as great with the former as with the latter compound (by measuring the percentage of the administered dose excreted as $^{14}CO_2$ over a two hour period). Thus O-demethylation by rat liver is predominantly 4-O-demethylation, as in the human.

It is not possible to judge whether amine oxidation (to D.M.P.E.A.) preceded demethylation or not in Sargent's experiment, since monoamine oxidase inhibition was not employed .

Conclusion:

Demethylation of D.M.P.E. by human liver appears to occur predominantly in the para position (4-O-demethylation).

4. <u>The inhibition of the demethylation of p-methoxyacetanilide (methacetin) by D.M.P.E.</u>

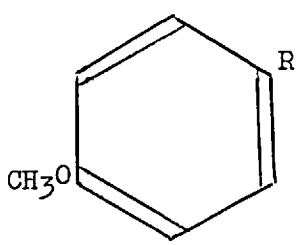
Introduction.

This experiment forms a link between the in vitro experimental

work described above and the in vivo work presently to be described. The original intention had been to study the demethylation of D.M.P.E. in vitro comparing schizophrenic and non-schizophrenic liver. The problems encountered while using human liver, and the difficulty in obtaining 'schizophrenic' liver have already been discussed.

The use of a chemical substance, the metabolism of which could be followed in vivo suggested itself as an alternative procedure. Demonstration that this chemical substance was demethylated by the same enzyme system as demethylated D.M.P.E. was clearly desirable.

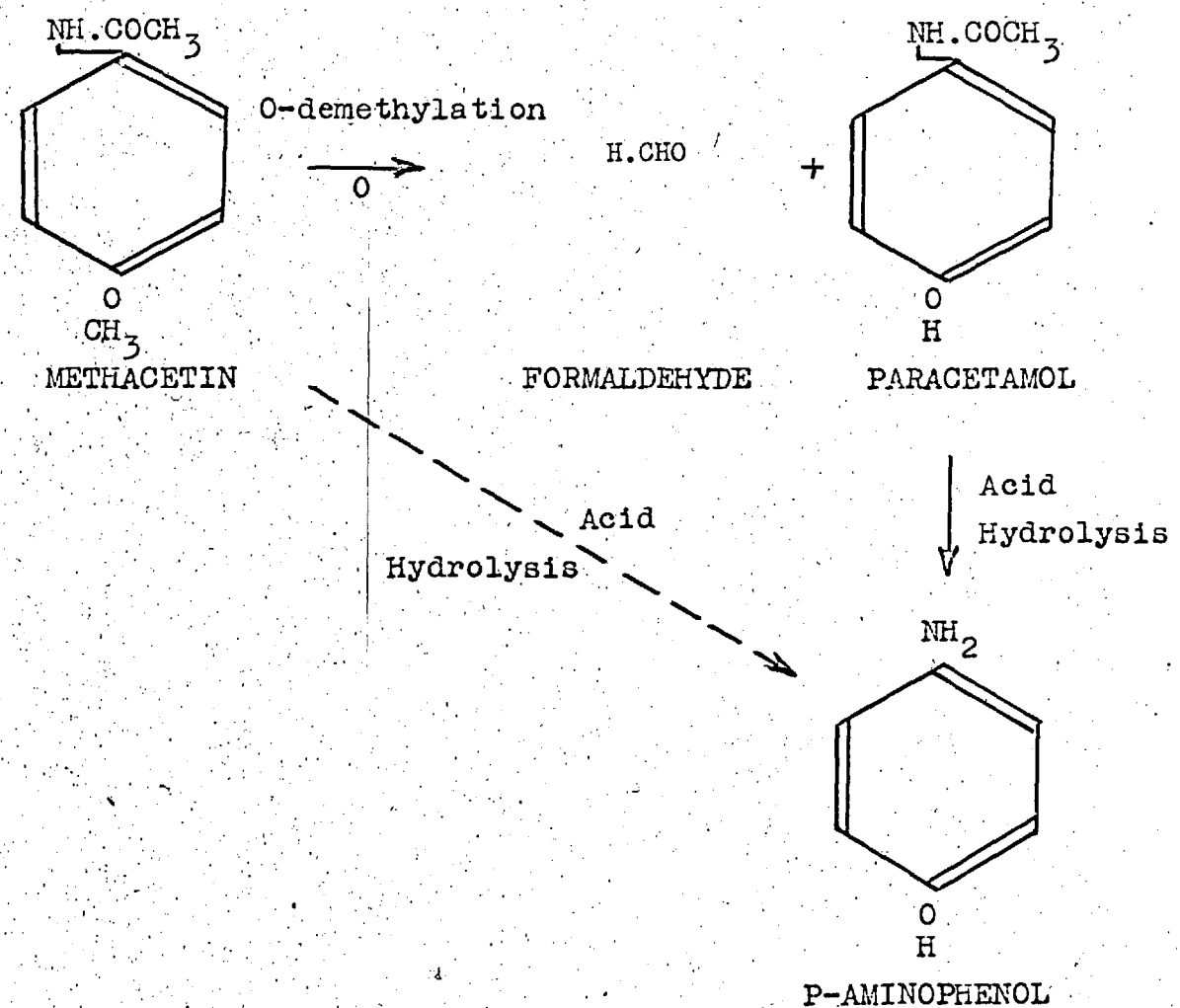
From Axelrod's work (1956) it seemed likely that substances of the form



were substrates for the same demethylating enzyme and that substrates with a more complex polycyclic structure (such as codeine) were demethylated by a second, separate, system.

In the experiment described here the effect of a 20-fold excess of D.M.P.E. is compared with the effect of a 20-fold excess of codeine on the demethylation of methacetin,

Figure 9. The Enzymatic & Chemical Breakdown of Methacetin to p-Aminophenol.



(p-methoxyacetanilide). What is measured is the yield of the demethylated product of methacetin according to the equation shown in Fig. 9.

The method of estimation of paracetamol is given in considerably more detail in a later section. It involves acid hydrolysis to para-aminophenol, the latter being brominated and reacted under alkaline conditions to give a blue coloured dye which can be measured spectrophotometrically.

The present experiment was complicated by a chemical property of methacetin whereby a small yield of p-aminophenol or something reacting like p-aminophenol in the colour reaction resulted from the acid hydrolysis of a sample of methacetin, as shown by the dotted line in Fig. 9. The coloured dye thus produced had the same spectrum in the visible range to that produced when a solution of paracetamol of 7% of the concentration of the solution of methacetin was similarly treated. The reason for this yield of colour is obscure but it was not due to contamination of methacetin with p-aminophenol since it did not occur until the methacetin had been acid hydrolysed. Nor was it due to contamination of methacetin with paracetamol since methacetin failed to yield the purple colour given by paracetamol with 1% aqueous ferric chloride, no matter how concentrated the methacetin solution used. Acid hydrolysis at increased pressure

(15 lbs/sq. in. for 90 minutes) did not increase the yield of colour. A specimen of phenacetin (kindly supplied by Dr. L. F. Prescott) behaved similarly.

In studies on urine presently to be described, this phenomenon does not matter because it can be shown that 85% or more of methacetin is converted to paracetamol; the amount of colour yielded by what is not converted to paracetamol must necessarily be small. In the present experiment, however, only a small amount of methacetin is demethylated. This means that the remainder is present unchanged in the enzyme solution in an amount greatly in excess of any paracetamol that may be formed enzymatically, the reverse of the situation in urine. No way of avoiding the phenomenon was found, so that quantities of paracetamol have no meaning in absolute terms, but comparison between flasks would still seem to be valid, the same amount of methacetin being added to them all, except the control flask, flask 5.

Materials:

The same human liver was used as in the previous experiments.

Methods:

Flasks were made up as follows, figures in columns 1 - 5 representing volume in ml.

FLASK NO.		1	2	3	4	5
1	Liver preparation	16	16	16	16	16
2	Nicotinamide 200 μmol) $MgCl_2$ 100 μmol)	8	8	8	8	8
3	T.P.N. 4 μmol	0.8	0.8	0.8	0.8	0.8
4	Sorensen's phosphate buffer pH 7.4 (0.5 mol)	3.2	3.2	3.2	3.2	3.2
5	32 μmol methacetin	4	4	4	4	-
6	0.64 m mol D.M.P.E.	-	8	-	-	-
7	0.64 m mol codeine phosphate	-	-	8	-	-
8	Phenelzine 4.8 μmol	1	1	1	1	1
9	Water	15	7	7	11	19
10	30% trichloracetic acid	-	-	-	4	-
TOTAL		48	48	48	48	48

Phenelzine was added (in a final concentration of 10^{-4} molar) in order to present destruction of D.M.P.E.; complete or near complete destruction of this compound by monoamine oxidase might have interfered with the competitive inhibition of the demethylating enzyme (depending on the degree to which the product of amine oxidation (3: 4 dimethoxyphenylacetic acid, D.M.P.A.A.) is a substrate for the demethylating enzyme).

After incubation at 37°C for two hours, 4 ml. of 30% T.C.A. was added to each flask, except flask 4, thus terminating the reaction and precipitating protein. The following additions were then made:-

	FLASKS				
	1	2	3	4	5
0.64 m mol [illegible]	8 ml	-	8 ml	8 ml	8 ml
0.64 m mol codeine phosphate	8 ml	8 ml	-	8 ml	8 ml
Water	-	8 ml	8 ml	4 ml	-

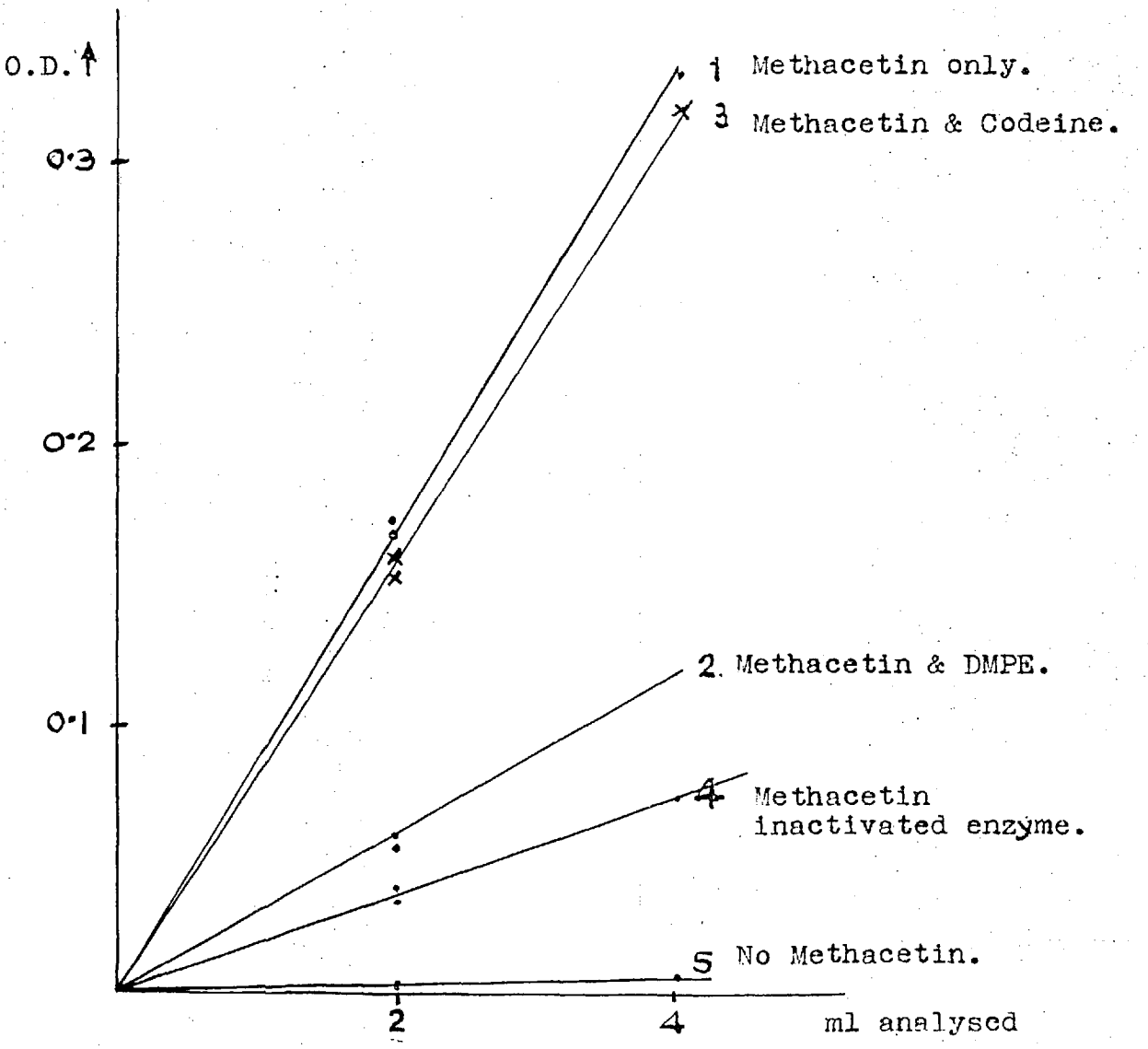
The final volume in each flask was 68 ml. The contents of each flask were then filtered through Whatman No.1 filter paper.

For acid hydrolysis of paracetamol to p-amino phenol, Brodie and Axelrod (1949) used a mixture of 1 part concentrated HCl to 4 parts neutral (or slightly alkaline) solution. Therefore, in the next stage of the present experiment 17 ml. of concentrated HCl was added to each flask (and acid hydrolysis carried out for 30 minutes in a boiling water bath).

The p-aminophenol resulting from this hydrolysis was estimated by adding duplicate portions of 20 ml. from each flask to a flask containing 24 G. K_2HPO_4. This was extracted with 200 ml. diethyl ether containing 1.5% V/V isoamyl alcohol. Of this, 80 ml. was transferred to a flask containing 12 ml. 0.01 N HCl. After shaking the acid phases were removed and each used to make up three tubes

Figure 10.

The effects of 20fold excesses of codeine & DMPE on the demethylation of Methacetin. O.D. is proportional to the amount of Paracetamol estimated to be present,(see text).



Number opposite each line corresponds to the flask number.

as follows:-

	Tube 1	Tube 2	Tube 3
Acid phase	4 ml	2 ml	2 ml
0.01 N HCl	1 ml	3 ml	3 ml
TOTAL	5 ml	5 ml	5 ml

Further additions to all three tubes were 1 ml. of 1% aqueous phenol and 1 ml. freshly prepared sodium hypobromite solution (made up by adding bromine water to 1 N sodium carbonate until the resulting solution was faintly yellow).

The final solution was shaken, and allowed to stand for 20 minutes at room temperature, after which the colour was read at 620 mμ in a Unicam 600 spectrophotometer.

Results.

	Flask 1	Flask 2	Flask 3	Flask 4	Flask 5
Tube 1	0.331	0.118	0.317	0.072	0.006
Tube 2	0.173	0.058	0.153	0.033	0.005
Tube 3	0.167	0.052	0.159	0.039	0.002

The results, which as shown graphically in Fig.10 show fair agreement with Beer's law.

Discussion:

Flasks 1 and 3. The addition of codeine in 20-fold excess appears to have little or no effect on the yield of paracetamol from methacetin. This can be seen in the close similarity in the yields

from flasks 1 and 3.

Flasks 1 and 2. A considerable effect is shown when a 20-fold excess of D.M.P.E. has been added, with a much smaller yield of paracetamol.

Flask 4. It was shown, by separate experiment, that an amount of methacetin equal to that used in this experiment (32 µmol), would, if completely extracted, have yielded more p-aminophenol on acid hydrolysis than that produced in any of these flasks. It would seem likely that the addition of trichloracetic acid and subsequent filtration must have removed a good deal of the interference.

Flask 4 was used as a check on this; this was identical in every way to flask 1, except that the 4 ml. of 30% trichloracetic acid was incorporated initially so that the enzymatic formation of paracetamol was prevented entirely.

The yield in flask 4 is lower, but not much lower, than that in flask 2. This would suggest that most of what was extracted as paracetamol and estimated as p-aminophenol from flask 2 was not formed enzymatically, but was derived non-enzymatically from methacetin by acid hydrolysis. The same cannot be said of flasks 1 and 3 where amounts of paracetamol are greatly in excess of that in flask 4, suggesting enzymatic paracetamol production on a larger scale.

Flask 5, was one to which no methacetin was added. Paracetamol

yields can be seen to be negligible. The liver was thus not contaminated with paracetamol to a detectable extent - a situation which might have come about as a result of phenacetin or paracetamol administration prior to death.

Conclusion:

The results of this experiment are consistent with the statement that, while D.M.P.E. and methacetin appear to be demethylated by the same enzyme in human liver, codeine and methacetin are demethylated by different enzymes.

Concluding Observations on O-demethylation experiments.

Although it was possible to obtain results with the techniques described above, such techniques have considerable difficulties among which a general sluggishness of activity as indicated by low formaldehyde yields was the most important. One liver was found which behaved well and from which experimental results were obtained. Other livers, while not sluggish, yielded a troublesome opalescence thought to be due to the presence of fat in homogenates.

The greatest obstacle to progress was, however, the difficulty experienced in obtaining post-mortem material from schizophrenics.

EXPERIMENTAL SECTION : 2

STUDIES OF O-DEMETHYLATION IN VIVO.

Introduction : The pharmacogenetic approach.

It has been mentioned on the section on the preparation of tissue homogenates that difficulties were obtained in obtaining fresh liver from schizophrenics (both at operation and at post-mortem).

One cannot assume that what have been called the schizophrenias are aetiologically homogeneous. Therefore to obtain a sizeable series of livers seemed obligatory since one could not extrapolate from one or two negative or one or two positive findings. The practical problem was thus multiplied.

A pharmacogenetic approach offered a means of circumventing this difficulty.

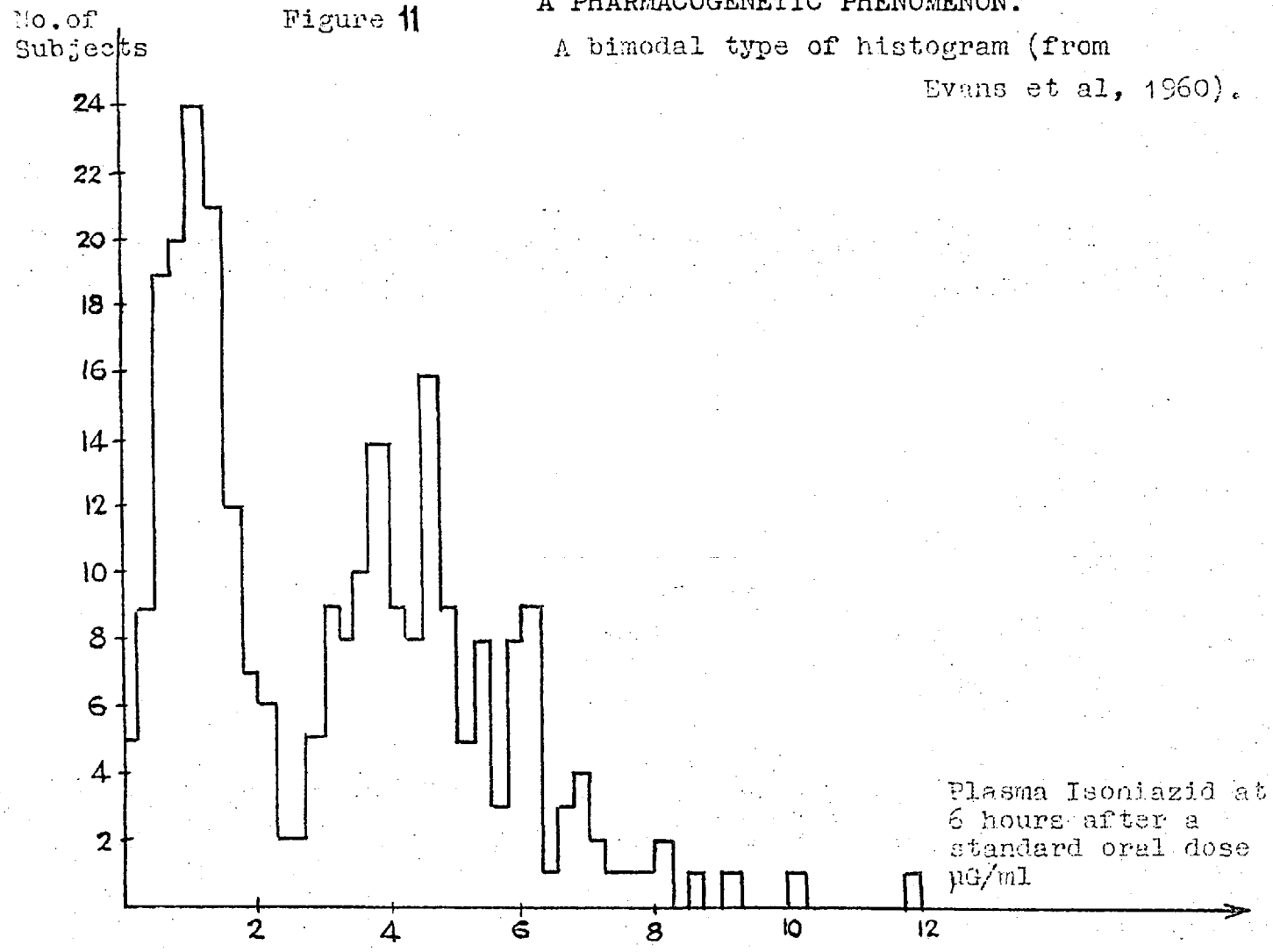
Vogel (1959) introduced the term pharmacogenetics which is defined as the study of genetically determined variations in animal species which are revealed by the effects of drugs. Thus an individual's glucose-6-phosphate dehydrogenase deficiency can be shown by administrating primaquine and precipitating a haemolytic anaemia; pseudocholinesterase deficiency can be demonstrated by showing an unduly prolonged apnoeic response to suxamethonium.

A genetic difference can also be shown to exist in a less dramatic way, namely by a study of the metabolism of administered substances. Thus, if a standardised dose of isoniazid is given to

Figure 11

A PHARMACOGENETIC PHENOMENON.

A bimodal type of histogram (from Evans et al, 1960).



subjects and the plasma concentration of isoniazid six hours later is plotted as a distribution histogram, a bimodal pattern results (Evans et al., 1960) as shown in Fig. 11.

Subjects with low values on this histogram can be shown (by in vitro studies) to have a liver enzyme capable of the rapid acetylation of isoniazid. Subjects with high values (corresponding to the right hand peak) can be shown to have a liver enzyme capable of acetylation at a slower rate.

Furthermore, family studies show that slow inactivation of isoniazid is governed by a gene having an autosomal recessive character. Of Europeans 56% belong to this slow inactivation category which may be designated by the genotype SS (S = slow, R = rapid). Hence 44% of Europeans are either RR or RS and in either case they are rapid inactivators of isoniazid.

Evans and White (1964) have shown by looking at a series of patients each of whom was studied both in vivo (using a drug administration test) and in vitro (by measuring enzyme activity on a liver biopsy specimen), that the results of the latter may be predicted invariably by a study of the former, thus rendering in vitro testing uninformative in an individual previously phenotyped by a drug test.

The suitability of schizophrenia for a pharmacogenetic approach.

Schizophrenia seemed suitable for study pharmacogenetically using

in vivo methods for the following reasons:-

a) that it is a common condition (so that the already mentioned need for a sizeable survey can be met without much difficulty).

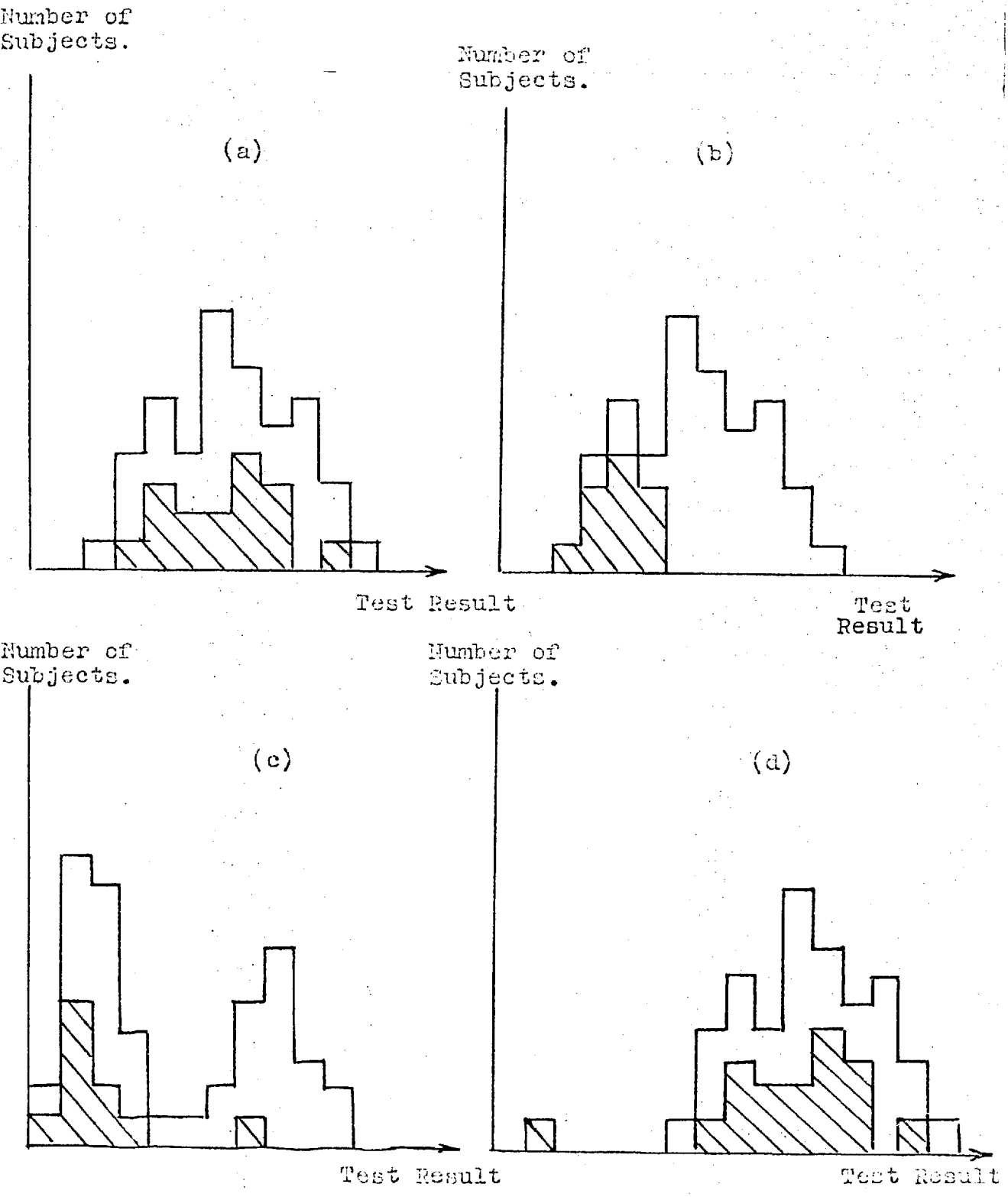
b) that biopsy material is not likely to be available during (nor post-mortem material immediately after) acute, well documented schizophrenic illness.

Disadvantages of in vivo studies: a) In vivo studies must necessarily blur the situation slightly. Thus in Fig. 11 , part of the observed difference in plasma level between one subject and another will be due to slight individual variations in the rate of absorption of the drug from the intestine.

Subjects at the antimode cannot always be allocated to one or other phenotype even with family studies; they may for example, be rapid inactivators who absorb the drug slowly or slow inactivators who absorb the drug rapidly. This example illustrates absorption as the important variable but other individual differences can also be predicted. Thus, differences in such factors as renal clearance and extra cellular fluid volume will occur and these also will effect the plasma level to some extent.

b) In many instances the in vivo procedure will involve transformations carried out by more than one enzyme. Minor difference in the activities of other enzymes may mask important genetic variability in the single enzyme that it is desired to study.

Figure 12 (a-d) Some of the results that might be obtained when a drug test is administered to two groups of subjects (results for one group are cross hatched).



c) Figure 11 was derived from 267 subjects, and test results derived from a small number of subjects would simply not have given conclusive evidence of the phenomenon it illustrates, i.e. bimodality.

This is not to say that a conclusively abnormal result could not be obtained in a single subject by an in vivo pharmacogenetic test. Whether this could happen would depend on the characteristics of the histogram obtained in normal subjects for the test in question.

<u>The possible results of the application of a drug test administered to two groups, one of normal subjects, the other of schizophrenics.</u> First, one might obtain the "null hypothesis" result shown in Fig. 12 (a). Alternatively, one might find that the 'patient' group fell at one or other end of the distribution curve for normals (Fig. 12 (b)). The type of pattern shown in Fig. 12 (b) was found by Harris et al., (1960) when they measured cholinesterase levels using acetyl choline as substrate in individuals (designated by the hatched area) showing sensitivity to suxamethonium.

Alternatively a bimodal pattern might occur, with or without a random distribution of results for schizophrenics throughout the histogram. Fig. 12 (c) shows a non-random distribution.

An example of this phenomenon is seen in work by Harris et al. (1949) which has since been confirmed by others. A polymorphism exists such that if a histogram is constructed in which a measurement of the most dilute solution of phenylthiocarbamide (P.T.C., a goitrogen) which a subject can taste is plotted for a random sample of the population as a histogram, a bimodal type of distribution curve is produced.

If a similar histogram is constructed for a group of subjects with nodular goitre more 'non-tasters' of P.T.C. will be found in this group than would be predicted on the null hypothesis that nodular goitre and P.T.C. status are unrelated. The distinction is not an absolute one, for certain of the patients with nodular goitre are ' tasters ' of P.T.C. and a considerable proportion of the normal population are 'non-tasters.'

As schizophrenia cannot be assumed to be homogeneous one also has the possibility shown in Fig. 12 (d).

This situation can be illustrated by reference to acatalsia. In this condition, very rare outside Japan, absence of the enzyme catalase is associated with oronasal sepsis. The enzyme is absent from the tissues of the oral and nasal cavities and from red cells (Takahara, 1963). Acatalasics are homozygous for a gene having a recessive autosomal character.

If one were to take a Japanese population and estimate red

cell catalase activity in i) normals and ii) patients with severe oronasal sepsis, and make a plot of enzyme activity against numbers of subjects, a graphical result similar to that shown in Fig. 12 (d) could result. This shows normal enzyme levels in both groups except for one of the group ii (an acatalasic) who would, in this instance, have zero enzyme activity. This subject alone among subjects designated as having "severe oronasal sepsis" would be acatalasic, indicating the heterogeneous nature of the group. It would not be unreasonable to anticipate similar heterogeneity in that group of conditions now designated as 'schizophrenia.'

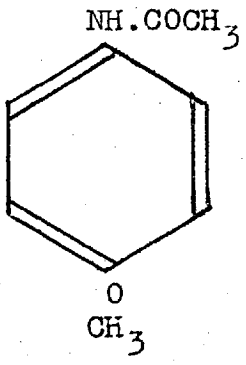
This argument can be extended in a different direction, namely that if deficiency of demethylation or excess of methylation exist as biochemical entities then a search for such abnormalities among schizophrenics is, on the evidence discussed in the literature survey, particularly worthwhile even if these entities have no direct connection with the abnormalities causative of schizophrenia.

METHACETIN TESTING

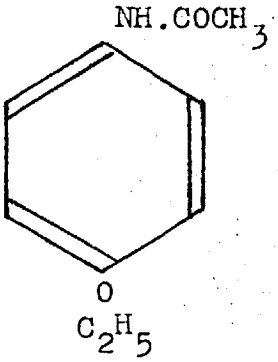
Introduction. The substance methacetin was chosen as being suitable for use in a pharmacogenetic investigation into the demethylation

SOME FORMULAE OF RELEVANCE TO THE METHACETIN STUDY.

Fig. 13(a). Methacetin & Phenacetin.

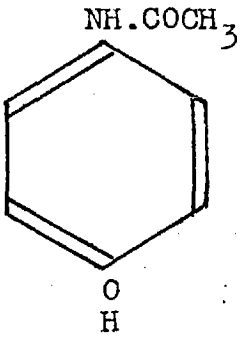


METHACETIN

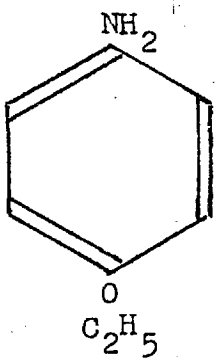


PHENACETIN

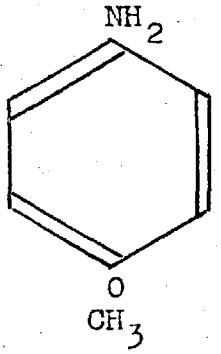
Fig. 13(b). Paracetamol, p-phenetidin & p-methetidin.



PARACETAMOL

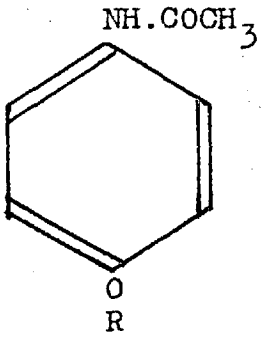


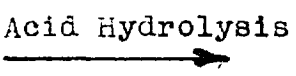
p-PHENETIDIN

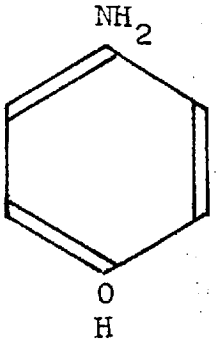


p-METHETIDIN

Fig. 13 (c). The acid hydrolysis of paracetamol & its conjugates.







R=H, or sulphate or glucuronide

p-AMINOPHENOL

hypothesis.

Methacetin is the methyl analogue of phenacetin (see Fig 13 a). Brodie and Axelrod (1949) examined the urine collected for 24 hours from human subjects after an oral dose of 1 - 2 G. of phenacetin and found that it contained 78% of the administered substance converted to free or conjugated N-acetyl p-aminophenol (paracetamol) ; of this 78% an average of 3.6% was free and 74.4% conjugated; 0.27% of the drug was excreted unchanged, and 0.1% in the form of p-phenetidin (see Fig. 13 b).

More recently Jagenburg and Toczko (1964) have isolated a new metabolite of phenacetin, s-(1-acetamido-4-hydroxyphenyl cysteine). In addition an acetyl derivative of this new metabolite may also be formed in low yield. These two metabolites account for only 2% approximately of the administered dose.

Methacetin, a substance with the same antipyretic and analgesic properties as phenacetin (May, 1921; Pharmaceutical Journal, 1889), is dealkylated at the same rate as phenacetin by the dealkylating enzyme system studied by Axelrod (1956). It seemed reasonable to suppose that the metabolism of both substances would be similar, that is that initial demethylation of methacetin (yielding paracetamol) would be followed by conjugation with glucuronide and inorganic sulphate (and possibly also with the formation of small quantities of the recently

identified conjugates mentioned in the last paragraph).

A detailed estimation of the amounts of sulphate and glucuronide formed in the metabolism of methacetin does not appear to have been carried out, but this information is available for paracetamol (Cummings et al., 1967), the demethylated derivative of methacetin.

Preliminary experiments to determine the validity of various laboratory procedures employed in methacetin testing.

In the present study what has been measured is the total excretion of demethylated metabolites i.e. of free and conjugated paracetamol, in a precisely timed collection of urine, after an oral dose of methacetin.

The method of determination of total (free and conjugated) paracetamol. The method closely followed the method of Brodie and Axelrod (1948). Where a specimen of urine containing a particularly high concentration of metabolites required analysis, only 1 ml. of urine (or very rarely even less) was used instead of 2 ml., with an additional 1 ml. of water.

In this way the Unicam values were kept within the range for which the parallel lines had been calculated as having validity (v.i). With very dilute urine samples as much as 5 ml. of urine was

used with a reduction in the amount of water added from 13 to 10 ml. In over 70% of cases, however, 2 ml. of urine was used for the analysis, as in the following account.

Method:

To 2 ml. of urine were added 13 ml. water and 3 ml. concentrated HCl, in a universal container. Duplicates were set up in all cases. The containers were firmly stoppered and subjected to autoclaving at 15 lbs/sq. in. for 1½ hours. No loss of volume occurred during this procedure.

5 ml. of the hydrolysate was added to 5 G. K_2HPO_4 in a stoppered flask, which was cooled in an ice bath.

50 ml. of diethyl ether (containing 1.5% V/V isoamyl alcohol) was added and the flask was shaken on a shaking apparatus for 10 mins. Exactly 20 ml. of the ether phase was then pipetted into a flask containing 3 ml. 0.01 N HCl.

The ether phase and the acid were shaken together for 10 mins and most of the ether decanted, the fluid remaining being transfered into a small centrifuge tube. Any ether remaining was then removed with a Pasteur pipette.

1 ml. of the acid phase was placed in a centrifuge tube, and 4 ml. 0.01 N hydrochloric acid was added bringing the volume up to 5 ml.

To this were added in turn (a) 1 ml. of freshly prepared

1% aqueous phenol and (b) 1 ml. of freshly prepared sodium hypobromite solution. This latter solution was prepared by adding bromine water to normal sodium carbonate until the shaken solution was slightly yellow.

The final solution was mixed, allowed to stand for 20 minutes at room temperature and read at 620 mμ in a Unicam 600 spectrophotometer.

Notes on this procedure:

1. Acid hydrolysis. This not only splits conjugates, but removes the acetyl side chain (see Fig. 13c), yielding p-aminophenol. It is the p-aminophenol that is estimated.

2. Standard solutions of p-aminophenol had to be made up in acid conditions for in neutral or alkaline solution this substance is unstable. With the addition of acid to K_2HPO_4 as occurred prior to the adding of ether in the procedure described above a maximum pH of 7.5 was produced, and heat was evolved. It was thought prudent to carry out this addition with the flask resting in an ice-bath.

Standard solutions of p-aminophenol kept for several weeks without deterioration (as judged by their behaviour in the colour reaction) if kept at -20°C (frozen) and allowed to thaw only briefly when required for use.

3. Shaking. Experiment showed that prolongation of the two

10 minute shaking periods failed to increase the yield.

4. The colour reaction. As far as the colorimetric side of the procedure was concerned, only a very small amount of bromine was required for the production of maximum colour. (Bromine water was kept in a bottle in which free bromine was present; the bottle was shaken and allowed to settle before use).

The period of 20 minutes for development of colour was more than adequate; after 15 minutes negligible further increase in colour density occurred. The optical density of the final solution remained constant for an hour or two but a fall was readily detectable in solutions kept overnight.

5. Limits of error. The parallel lines experiment was carried out at an early stage and agreement of duplicates within 10% was permitted in this experiment. When practice had been obtained with the procedure agreement within 3% was usually found and duplicates failing to agree by a margin of more than 5% were repeated.

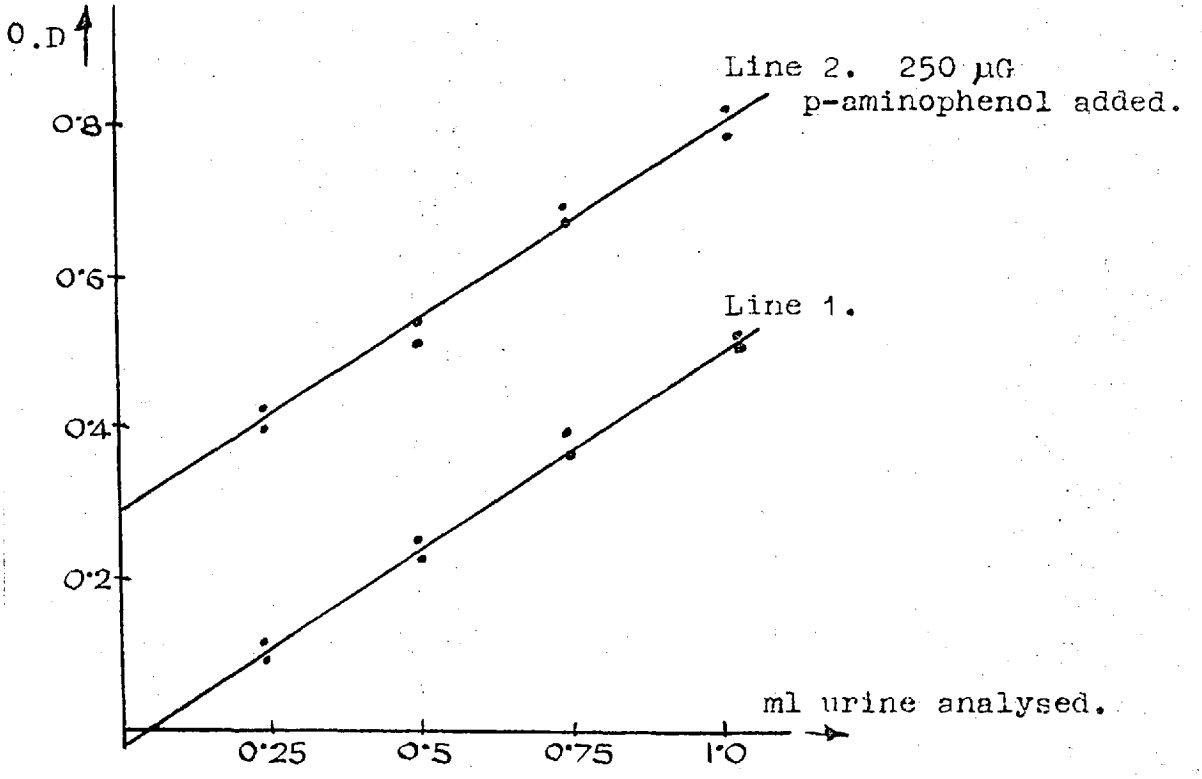
The parallel lines experiment. The experiment was designed to show that no constituent of urine interfered with the method of estimation of paracetamol.

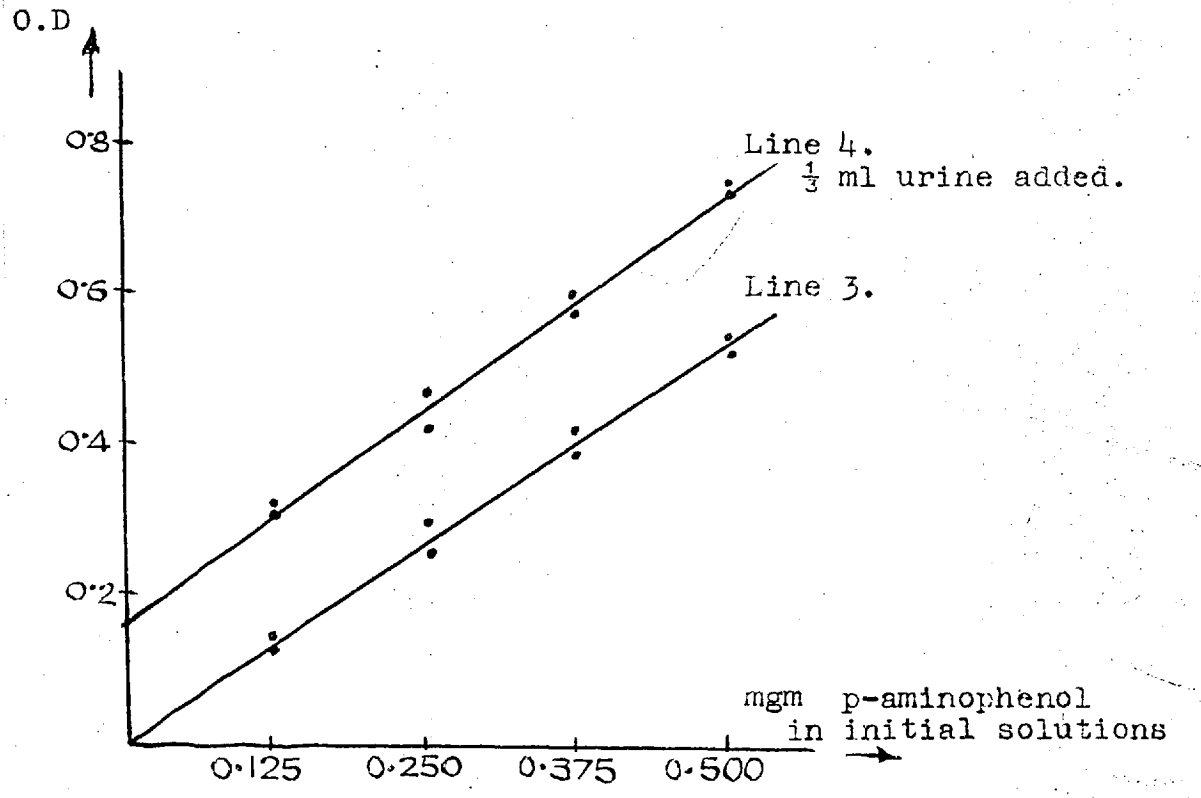
Materials:

1. Urine was collected from a subject who had received an oral dose of 1 G. of methacetin and who had thereafter collected all

Figure 14.

PARALLEL LINES EXPERIMENT FOR TOTAL PARACETAMOL ESTIMATION IN URINE.





urine (540 ml.) for four hours.

2. The standard solution of p-aminophenol contained 250 μG per 0.5 ml. 0.01 N HCl.

Method:

The method used for estimating paracetamol and its conjugates has been described above.

A total of 32 universal containers were prepared, eight for each of four lines, the material used for each line being as follows:-

Line 1. Serial dilutions of urine.

Line 2. " " " " with addition of a constant amount of authentic p-aminophenol (250 μG).

Line 3. Serial dilutions of a stock solution of p-aminophenol.

Line 4. " " " " " " " "

with the addition of a constant amount of urine (1/3 ml.)

Results:

An account of this method of evaluating interference has been given in some detail elsewhere in connection with formaldehyde estimation.

The values obtained in the present experiment are depicted in Fig. 14.

In order to avoid repetition, suffice it to summarise the results of the statistical calculations:-

1. All lines have slopes differing significantly from zero with

$p < 0.0001$ in all cases.

2. The slope of line 1 (0.529) did not differ significantly from the slope of line 2 (0.528) ($t = 0.019$).

3. The slope of line 3 (1.008) did not differ significantly from the slope of line 4 (1.126) ($t = 1.85$).

4. The intercepts of lines 1 and 3 did not differ significantly from zero.

5. The intercept of line 2 (0.268) and of line 4 (0.165) differed significantly from zero, with $p < 0.001$ in both cases.

6. The intercept for line 4 (0.165) corresponded to the addition of 1/3 ml. of urine to all tubes. The expected intercept from 1 ml. of urine would therefore have been $3 \times 0.165 = 0.495$. This agreed satisfactorily with the value obtained from line 1 for the O.D. due to 1 ml. of urine : 0.507.

7. The intercept for line 2 (0.268) corresponded to the addition of 250 μG. p-aminophenol to all tubes. This agreed satisfactorily with the value obtained from line 3 for 250 μG. p-aminophenol (0.266).

Discussion:

It was considered an impracticable proposition to repeat this parallel lines procedure on urine from each schizophrenic; yet strictly this would need to be done if interference from drug metabolites were to be excluded. What has been done is to

demonstrate that pretest urines from schizophrenics yielded very low Unicam readings when taken through the same procedure as test samples; and that prior administration of one or other of various phenothiazine drugs had no observable tendency to influence results (v.i.).

Conclusion:

The parallel lines experiment demonstrates that no constituent of the urine interfered with the colour reaction.

Recovery of p-aminophenol. This was estimated by comparing the results in line 3 with results obtained when standard amounts of p-aminophenol in 0.01 N HCl were used in the final stages of the procedure only - i.e. the part in which to a total of 5 ml. of 0.01 N HCl were added the reagents described above; the percentage recovery was found to be 87.6%. Brodie and Axelrod (1948) give the partition of p-aminophenol between the ether and neutral phase (K_2HPO_4) as being 85% in favour of the ether phase : in the present procedure the size of these phases and their composition is identical to that used by Brodie and Axelrod. The figure obtained here suggests that extraction back into acid from ether must be virtually complete and that losses during acid hydrolysis must be very small.

Calculation of the amount of methacetin from which metabolites had been derived. From line 3, derived from standard solutions

of p-aminophenol, an original amount of 500 μG of p-aminophenol (in the Universal container) yielded a Unicam reading of 0.533.

This would have been derived from an amount of methacetin equal to 756.8 μG i.e. $\frac{500 \times 165.2}{109.15}$ μG, 165.2 being the molecular weight of methacetin, and 109.15 that of p-aminophenol.

Suppose a sample of urine, total volume V ml. contains metabolites derived from X mgm. methacetin and that these are solely responsible for the final colour production then -

$$\text{concentration of metabolites} = \frac{X}{V} \text{ mgm/ml.}$$

If 1 ml. of urine containing metabolites derived from 756.8 μG methacetin gives a final Unicam reading of 0.533, then 1 ml. of urine containing metabolites derived from $\frac{X}{V} \times 1000$ μG of methacetin gives a final Unicam reading (U) = $0.533 \times \frac{(X) \times 1000}{(V) \times 756.8}$

Hence $(X) = \frac{U \times V}{0.533} \times \frac{756.8}{1000} = U \times V \times 1.42$, in mgms.

Where 2 ml. urine is analysed, this formula becomes

$$X = U \times V \times 0.71 \text{ mgms.}$$

Interference from substances in the urine contributing to the colour measured. Urines were taken from six normal and all schizophrenic subjects prior to administration of methacetin; the urines were processed in exactly the same way as test samples,

and Unicam readings were compared with those obtained using urine collected for four hours after methacetin administration. Unicam readings were very low - of the order of 0.001 and 0.002. Corrections for urine blanks were not made in normal subjects or in schizophrenics (except in a cumulative excretion study presently to be described).

A limitation in the value of the methacetin test as shown by studies of the excretion of methacetin and paracetamol.metabolites.

Experiment 1. A 24-hour collection of urine was made by a healthy male subject, who then carried out the methacetin test procedure (v.i.) in the usual way, consuming the dose of methacetin appropriate to his body weight:- 542 mgms.

Collections were made into a series of containers, (which contained 1 ml. 3% sodium azide as a preservative) as follows:-

1.	0 - 1 hr.	6.	6 - 10 hrs.
2.	1 - 2 hrs.	7.	10 - 24 hrs.
3.	2 - 3 hrs.	8.	24 - 30 hrs.
4.	3 - 4 hrs.	9.	30 - 48 hrs.
5.	4 - 6 hrs.	10.	48 - 54 hrs.

Normal meals were taken, except for a pretest fat free breakfast. An aliquot, taken from the 24-hour "pre-methacetin" collection of 975 ml. was estimated to contain 1.66 mgms. methacetin/l., (1.62 mgms. in all). This served as a "urine blank,"

and illustrates the low interference from substances in normal urine.

Values for cumulative total paracetamol excretion (expressed in the form of methacetin from which the metabolites were derived) were corrected for this "blank" value by using the formula:-

$$\text{Corrected Total} = \text{Actual total} - 1.62 \times \frac{\text{accumulated time}}{24}.$$

Results (expressed as mgm. methacetin).

TABLE VI : The Cumulative Excretion of Methacetin Metabolites.

Time in hrs.	0-1	1-2	2-3	3-4	4-6	6-10	10-24
Vol. Urine	59	38	43	127	103	167	680
Methacetin in mgm.	29.5	56.2	60.1	47.7	92.0	86.8	92.5
Cumulative total	29.5	85.7	145.8	193.5	285.5	372.3	464.8
Corrected total	29.4	85.6	145.6	193.2	285.1	371.6	463.2

	24-30	30-48	48-54
Vol. Urine	310	745	485
Methacetin in mgm.	13.0	4.0	0
Cumulative total	477.8	481.8	481.8
Corrected total	475.6	478.6	

The total for 48 hrs., 478.6 mgm. represents 88.3% recovery; 85.5% of the administered dose was excreted in the first 24 hours.

This conclusion is important because it demonstrates that what is being studied is by far the most important metabolic transformation. Other metabolites, for example unaltered methacetin and p-methetidin must necessarily only be present in small amounts.

Experiment 2. An experiment similar to that described above was carried out on the same subject using an equimolecular amount of paracetamol (496 mgms.), two weeks after methacetin had been consumed.

The results were corrected for urine 'blank' by the method described for methacetin : on this occasion a pretest 24-hour specimen 'yielded' the equivalent of 0.64 mgm/L of paracetamol in a sample of 1100 ml - 0.7 mgm/24 hours.

Results (expressed as mgms. of paracetamol).

TABLE VII: The Cumulative Excretion of Paracetamol Metabolites.

Time in hrs.	0-1	1-2	2-3	3-4	4-6	6-10	10-21	21-29	29-48	48-54
Vol. Urine	49	51	68	87	114	275	920	750	1100	285
mgms. p'ol.	46.2	66.8	60.6	44.5	62.2	64.0	74.0	26.7	3.7	0
Cumult. totals	46.2	113.0	173.6	218.1	280.3	340.3	414.3	441.0	444.7	444.7
Corrctd. totals	46.2	112.9	173.5	218.0	280.1	340.0	413.7	440.1	443.3	

This gives a recovery of 89.4% in 48 hrs.

A comparison of the results of experiments 1 and 2. In Table VIII the methacetin figures have been converted into mgms. of paracetamol by multiplying them by the ratio:-

$$\frac{\text{Mol. wt. paracetamol}}{\text{" " methacetin}} \quad (=0.915)$$

TABLE VIII : A Comparison of the Cumulative Excretion of Equimolecular Amounts of Paracetamol and Methacetin.

Time (hrs)	0-1	0-2	0-3	0-4	0-6	0-10	0-48
Methacetin	26.9	78.3	133.0	176.8	260.1	340.0	436.3
Paracetamol	46.2	112.9	173.5	218.0	280.1	340.0	443.3

If Mu_∞ is the amount of paracetamol plus paracetamol metabolites which are ultimately excreted, and the cumulative amount excreted at time t is Mu then

$$Mu_\infty - Mu = K_1 e^{-Kt}$$

where K_1 and K are constants (Cummings et al., 1967).

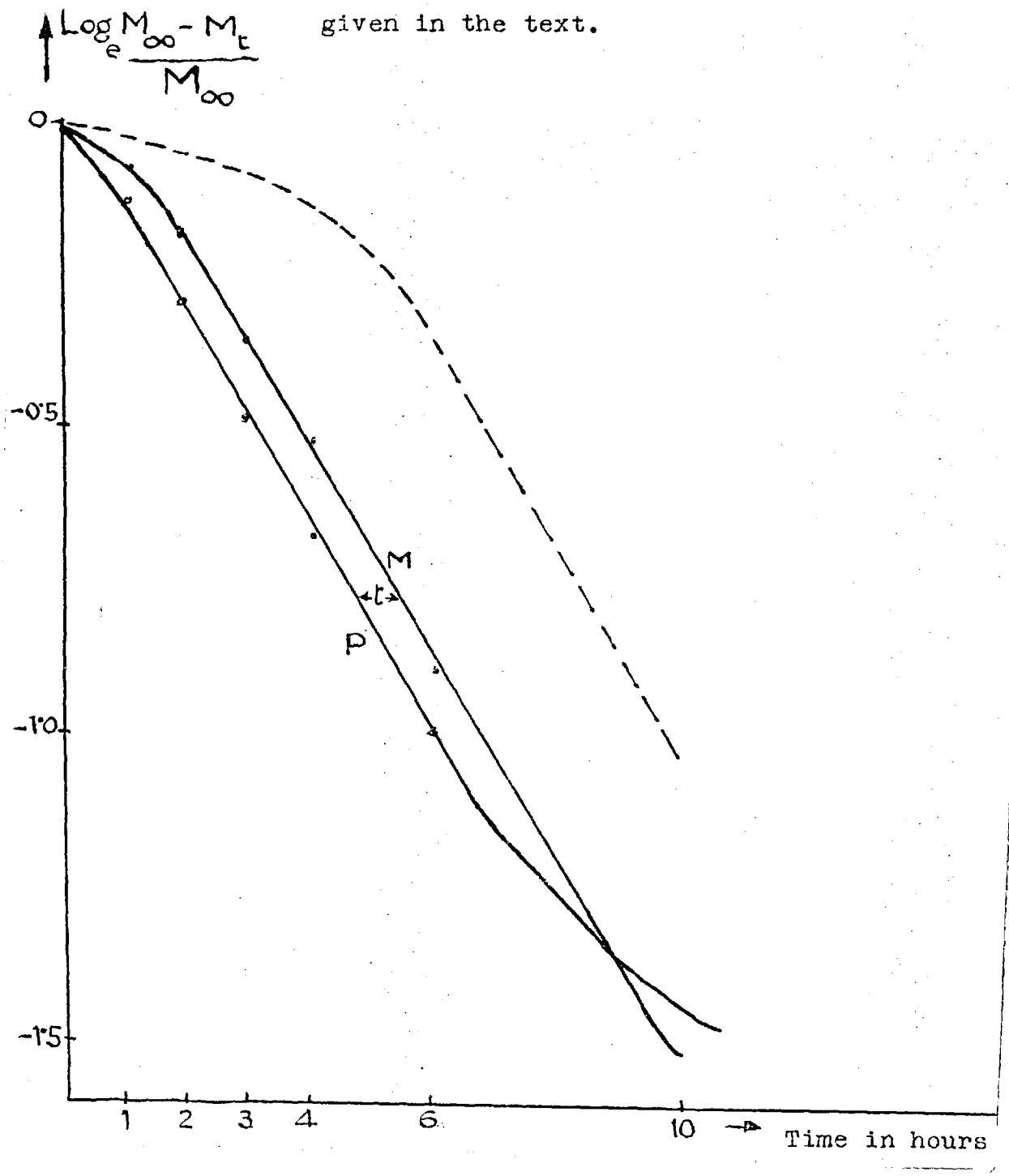
Hence $$\frac{Mu_\infty - Mu}{Mu_\infty} = \frac{K_1 e^{-Kt}}{Mu_\infty}$$

$$\log_e \frac{Mu_\infty - Mu}{Mu_\infty} = (\log_e K_1 - \log_e Mu_\infty) - Kt$$

This theoretical relationship is linear, but the graphs obtained are only linear over that period of time which follows the moment of maximum excretion. This will clearly not be the moment of ingestion of paracetamol (because it has to be absorbed), nor the moment of ingestion of methacetin (because it has to be

Figure 15.

Cumulative Excretion of METHACETIN (M) and PARACETAMOL (P) METABOLITES in the same subject. The other abbreviations are given in the text.



both absorbed and demethylated).

Fig. 15 shows the data of Table VIII plotted by the Sigma minus method used by Martin (1967).

$\text{Log}_e \frac{Mu_\infty - Mu}{Mu_\infty}$ is plotted against t.

The values from which Fig. 15 is constructed were derived by estimating the ratio $\frac{Mu_\infty - Mu}{Mu_\infty}$ from Table VIII and are as follows:-

Cumulative Time (in hrs.)	1	2	3	4	6	10
Methacetin : Ratio	0.937	0.820	0.695	0.595	0.404	0221
:Log_e(ratio)	-0.065	-0.198	-0.364	-0.519	-0.906	-1.510
Paracetamol : Ratio	0.895	0.745	0.609	0.509	0.368	0.238
:Log_e (ratio)	-0.111	-0.294	-0.496	-0.675	-1.00	-1.435

The graphs obtained for methacetin and paracetamol shows satisfactory linearity (see Fig. 15).

It is to be noted that the lines obtained on the 'sigma minus' plot are parallel, (with methacetin in the higher position (of later metabolite excretion). The parallelism is not fortuitous : it represents the total rate at which the free paracetamol, the paracetamol sulphate and the paracetamol glucuronide are being excreted from the body, those being metabolites common to both drugs.

Experiment 3. The finding of a more rapid excretion of paracetamol than of methacetin was confirmed in a study of 13 adult subjects : the subject described above, 9 healthy male volunteers and 3 healthy female volunteers.

Method:

The subjects first consumed a dose of methacetin under conditions which are specified in a later section, dosage (also specified later) being in proportion to metabolic active mass. The subjects collected urine for exactly four hours thereafter. A few weeks later the same subjects consumed an equimolecular dose of paracetamol under similar conditions, again collecting urine for exactly four hours.

Results:

Test Subject		Excretion in 4 hr. collection as % of administered dose of		Sign* P - M
		Paracetamol	Methacetin	
Males:	Subject in the above study	44.0	35.7	+
	Control No. 1	46.5	48.3	-
	No. 8	35.7	21.3	+
	9	39.6	30.5	+
	10	39.7	40.5	-
	17	45.3	39.1	+
	28	44.3	39.1	+
	31	62.5	50.8	+
	38	39.4	40.7	-
	39	47.4	46.1	+
Females:	1	35.3	27.7	+
	3	37.3	32.1	+
	6	48.0	40.5	+
	Mean:	43.5	37.9	

*Note : Paracetamol result greater than methacetin result score +; otherwise -.

Statistical Evaluation.

Using a one tailed sign test, the probability that P will

exceed M in 10 cases out of 13 is 0.046, i.e. the findings achieve statistical significance at the 0.05 level. The mean difference (P-M), 5.6% is somewhat lower in the whole group than in the subject who undertook cumulative collections (in whom P-M = 8.3). Discussion of the 3 experiments described above:

The distance between the lines of Fig. 15 can be accorded a value t (0.7 hrs. approximately). B. K. Grant, in a personal communication to the author has suggested that the value of t depends upon at least two factors - the relative rates of absorption of methacetin and paracetamol which could act to increase or decrease t (e.g. if methacetin is absorbed more slowly than paracetamol, t will be increased) and the time taken for demethylation which can only act to increase t; there is, unfortunately, no means of separating these two factors by experimentation on the human subject. If t consisted only of time needed for demethylation, the methacetin test could be judged to be a reasonably sensitive indicator of demethylating activity in the subject described above. Thus, an increase of t from 0.7 to 2.8 hrs. corresponding to a 4-fold reduction in the rate of demethylation would shift the methacetin line to the position shown by the dotted line in Fig. 15. It can be calculated that this line would give a methacetin test result of 20.0% which would place it outside the normal range.

On the whole, the mean difference between methacetin and paracetamol test results being less for other subjects than in the subject who made cumulative collections, the methacetin test should be judged as an insensitive one. In the three subjects handling methacetin more rapidly (in each case by a small margin) than paracetamol one must assume either that the latter drug was the more slowly absorbed; or that some incidental variation in absorption, metabolism or excretion was responsible for the result; or that an error was made at some stage in the test procedure.

It would seem from the data presented here that O-demethylation is not the rate limiting factor determining the excretion of methacetin metabolites in normal subjects. If it were, the lines of Fig. 15 would not be parallel but would diverge with increasing time.

The lack of O-demethylation as the rate limiting step is an undesirable property of methacetin as the test substance, although this property might not be present in the hypothetical subject with low enzyme activity. In the extreme case of a subject entirely lacking the enzyme, demethylation must necessarily be rate limiting and this abnormality would necessarily be shown up by the test.

In discussing the applications of pharmacogenetics to studies

of the type that are described here, a limitation of this method of approach to a process such as O-demethylation is apparent : if the substrate is one against which the demethylating enzyme is quick to act, then subsequent conjugation (e.g. with glucuronide or sulphate) at the free OH group is likely to occur for a considerable variety of such substrates, introducing unavoidable complication.

A detailed consideration of the subjects and methods used in methacetin testing.

1. Selection of normal subjects for methacetin testing. Volunteers were sought among University students and laboratory technicians, a method which attracted more male than female applicants; it also attracted subjects within the narrow age range 18 - 22. Older subjects were found not only to be less likely to volunteer, but also more likely to default. Subjects were mostly University students who would be unlikely to be suffering from gross physical disease, since routine medical examination (including urine testing) is carried out before acceptance into University.

2. Selection of acute schizophrenics for methacetin testing. In their paper in 1965, Bourdillon et al. refer to the fact that there was an excellent correlation between the diagnosis of Schneider positive non-paranoid schizophrenia and the excretion of pink spot material in the urine. Schneider positive symptoms

are symptoms stated by Schneider (1959) to be of first rank in determining the diagnosis of schizophrenia.

When methacetin testing was started, these results were available and the psychiatrist under whose care most of the cases were admitted undertook to look out for patients where the diagnosis was non-paranoid schizophrenia, with Schneider positive symptoms. In occasional cases where there was doubt about the diagnosis of schizophrenia, the methacetin test was carried out and the result discarded if the diagnosis of schizophrenia was subsequently abandoned, such cases being excluded from the present series.

In all other cases, patients were selected (by a psychiatrist who had contributed to the paper by Gourdillon), in whom no other diagnosis than that of schizophrenia had at any time been put forward.

Although some of these patients had been ill for a number of years, they were all in a shifting phase of their illness (and to this extent not chronic) and were usually tested within three days of admission to hospital (except for patients 1 - 6 who were taken off drugs while in hospital and then tested).

3. Dosage. Dosage was calculated according to the method of Evans et al. (1961), which is based on the concept of metabolic active mass, i.e. dose = k x (body wt)$^{0.7}$. All normal subjects

Fig. 16 PARACETAMOL or METHACETIN TEST

1. Subjects should on no account have taken any preparation containing paracetamol (PANADOL) or phenacetin (e.g. tabs. A.P.C., tabs. Codis Co.) in the 72 hours prior to the test. Please let us know the names of any drugs you have taken over the last month.

2. Subjects should consume a fat free breakfast on the morning of the test:-

   Grapefruit (optional). Toast with marmalade but no butter.
   2 cups of tea or coffee(normal amount of milk allowed).

3. Subjects should fast for 3 hours thereafter. Smoking is not allowed during this period (nor during the test). Water is allowed during this 3 hour period, to quench thirst.

4. It is desirable (but not essential) that the subject empties his bladder just prior to starting the test. This specimen is not, of course, passed into the container.

5. Three hours (or more) after breakfast the powder will be administered in hot tea (which has been strained to remove tea leaves. STIR THIS THOROUGHLY until no powder remains on the surface of the tea. Add milk and sugar to taste.

6. The subject should consume all the contents of the cup which will be inspected to make sure it is empty. The cup can be washed out, by taking a second cup of tea, but this is optional.

7. Nothing is allowed by mouth for 1½ hours after the test. A normal lunch may be taken after this interval and fluid should, after the first 1½ hours be taken freely.

8. All urine should be collected from the moment the drug is taken until exactly 4 hours after administration. At the time corresponding to 4 hours after administration the subject should empty the bladder fully, and include this sample in the container provided. This concludes the test.

9. The container should be kept in the coolest place available and handed in as soon as possible after the test is completed.

were weighed in everyday clothes, without shoes, with a subtraction for clothes of 5 lb. for men and 3 lb. for women. For a patient of either sex wearing pyjamas and a dressing gown, a subtraction of 2 lb. was made.

From the weights thus obtained and the above formula, the following dosage schedule was constructed:-

Body Weight (St./lb.)	Dose of Methacetin (mgm)	Body Weight (St./lb.)	Dose of Methacetin (mgm)
6.1 - 6.6	367	10.9 - 11.1	542
6.7 - 6.12	385	11.2 - 11.8	560
6.13 - 7.4	402	11.9 - 12.2	577
7.5 - 7.10	420	12.3 - 12.9	595
7.11 - 8.3	437	12.10 - 13.4	612
8.4 - 8.9	455	13.5 - 13.12	630
8.10 - 9.2	472	13.13 - 14.3	647
9.3 - 9.9	490	14.4 - 14.11	665
9.10 - 10.2	507	14.12 - 15.4	682
10.3 - 10.8	525	15.5 - 15.13	700, etc.

4. The methacetin test procedure (see Fig. 16). No departure from the test procedure was permitted in any case. Deviations of which the investigator was unaware(for example the comsumption of a breakfast containing fat which might have lead to delayed methacetin absorption) cannot be guarded against but one advantage of using

University students as controls should be their better than average intelligence. This should have made them aware that the rules laid down were purposeful and that departure from them was likely to prejudice the investigation. Methacetin was consumed in the form of a solution as in this form it will most rapidly be absorbed.

Paragraph 4, which requests the subject to empty the bladder prior to the test was not enforced rigidly; this was an unfortunate omission, because information could not subsequently be derived by studying the pH of the test urine or its creatinine content or its volume because some urine collected during the test was secreted before the test period started.

Collections were made into polythene containers, wide mouthed containers being made available to female volunteers. No preservative was used, but urine was prepared for acid hydrolysis immediately the test had been completed, an aliquot being put aside for future use, this being stored at -20°C.

5. Observations made on each subject and each urine collection. For each subject a note was made of sex, age and body weight. On each urine specimen the volume was measured and the total content of demethylated metabolites determined.

The data obtained is given in Tables XXIV - XXVb, p. 303 - 310

Age was taken as the age at the time of test (without

reference to the nearest birthday).

The dose was weighed out accurate to the nearest mgm., allowing an additional 1.5 mgm., this being the amount which remained acherent to the foil in which the methacetin was wrapped after as much as possible of the powder has been dislodged.

Volumes of urine were estimated to the nearest 10 ml. in measuring cylinders, at room temperature. Volumes falling on the half-way line between increments of 10 e.g. 395, 615, are recorded as such.

The methacetin test result:

This was expressed as the amount of methacetin from which the paracetamol and its conjugates recovered in the four hour collection were derived, this being expressed as a percentage of the administered dose.

6. Pink spot testing of the urine of acute schizophrenics (as described by Bourdillon et al., 1965, Method 2), was carried out on a urine collection made on the day before the methacetin test. For example, if a patient was admitted on Tuesday afternoon, a collection for pink spot testing was made on Wednesday and the methacetin test carried out on Thursday morning.

7. The methacetin testing of acute schizophrenics (and chronic mental hospital patients (v.i.)). The techniques used for testing volunteers were transferred without modification to acute

schizophrenics except that patients were instructed to empty the bladder just prior to the administration of the test substance, a procedure which enabled estimations of urinary creatinine, urinary pH and urinary volume to refer only to the four hour test period. The procedure reduced the number of times that the patient had to micturate during the four hour period (each act of micturition during the test period being an opportunity for something to go wrong).

Close supervision of patients during testing was carried out in all cases. This was done either by the author, or by a research nurse who had been trained in the test procedure, or by the staff of an observation side ward.

At the end of the four hour period all patients were instructed to empty the bladder as completely as possible. This was the stage at which co-operation was most necessary. Patients who expressed a desire to pass urine shortly before the end of the four hour period were asked to wait until the four hour period was completed and always succeeded in doing this.

8. Drugs and the methacetin test: Note was taken of medication prior to testing and of the probable length of time between cessation of medication and testing. In this connection one is forced to speak in terms of probable length of time, since the patient was not always able (and willing) to say when he had last taken tablets.

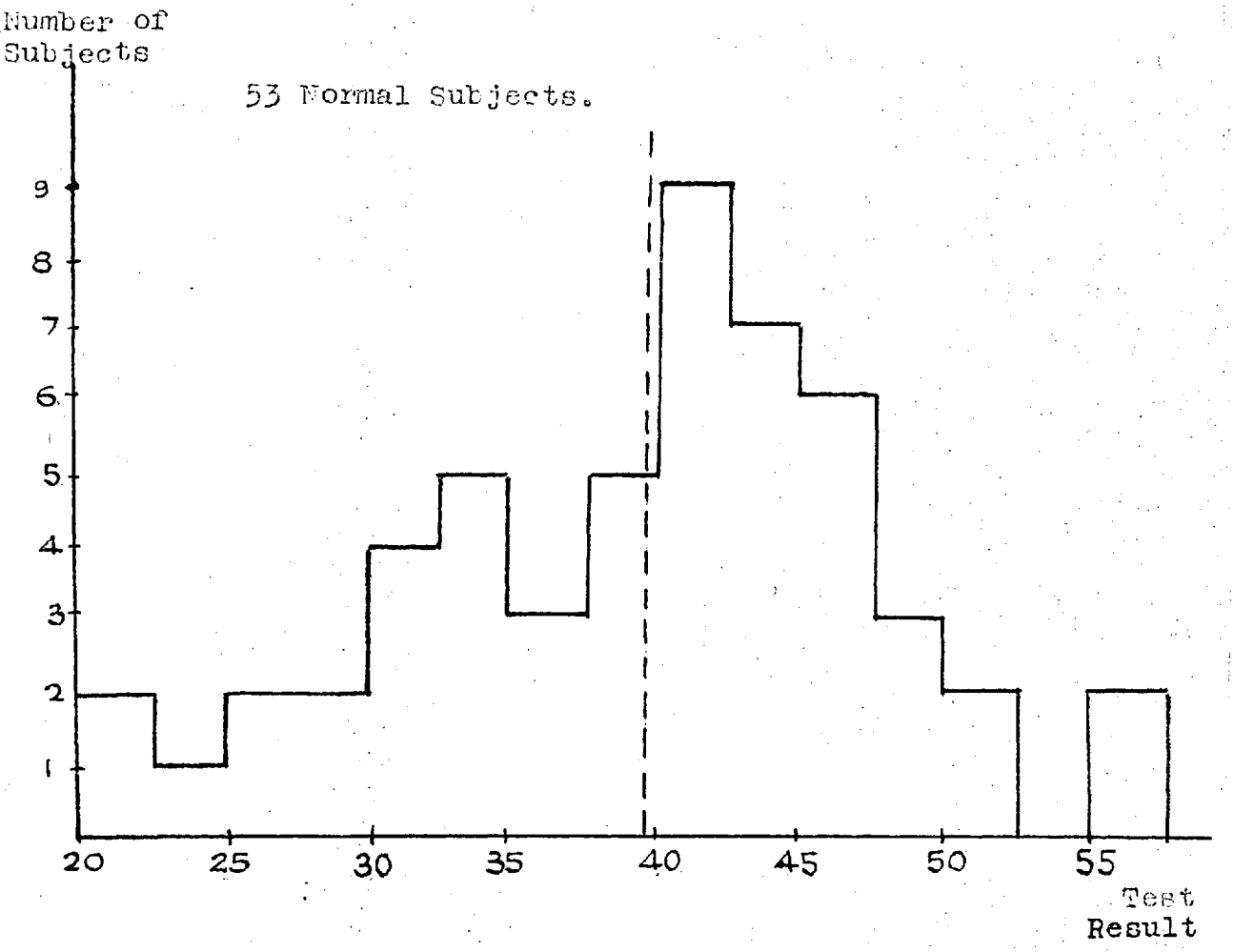
Preparations containing phenacetin or paracetamol in any form were withdrawn from the wards where tests were carried out. A pretest urine specimen was routinely examined for paracetamol by the usual methods always with negative results.

As far as hypnotics were concerned, these were seldom required. Barbiturates are well known for their ability to induce hepatic microsomal enzymes. Barbital and phenobarbitone have been particularly studied in this respect. Sodium amytal (200 - 400 mgm) of which was permitted, though seldom required in the present study, has received less attention. Henderson and Mazel (1964) showed in a study in rats that pretreatment with phenobarbital did not affect the demethylation of methacetin. However Uüch et al. (1965) did demonstrate an in vivo induction in rats of the enzyme responsible for the conjugation of paracetamol to paracetamol glucuronide, by phenobarbital.

The desirability of avoiding barbiturate administration was indicated to those clinicians helping with the present investigation.

Sodium amytal does not appear to interfere chemically with paracetamol estimation nor does it impair the quality of paper chromatography for pink spot (whereas phenothiazines do). Phenothiazine consumption prior to testing is considered separately in a subsequent section.

Figure 17 HISTOGRAMS of Methacetin Test Results.
The dotted vertical lines indicate mean values.



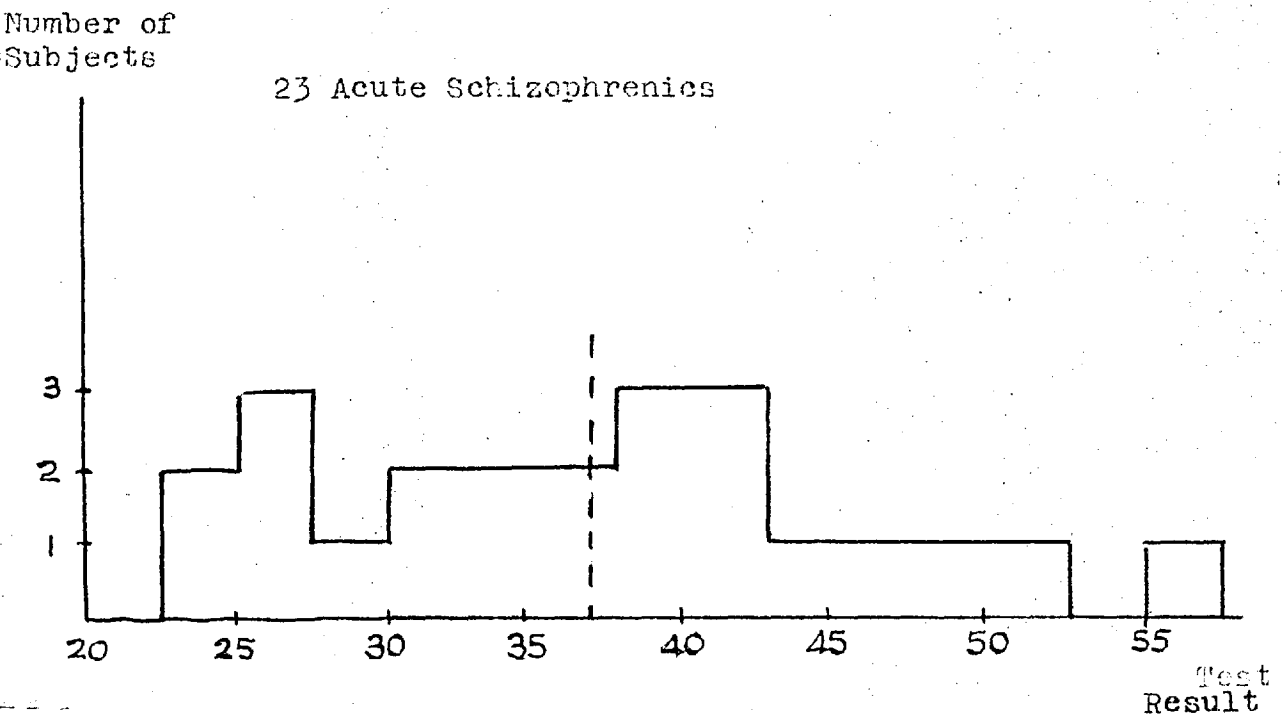


TABLE IX. Results for control subjects and acute schizophrenics.

	53 control subjects	23 Schizophrenics	t test for difference t	p
1. Methacetin test result	Mean 39.36	36.78		
	S.D. 8.10	8.86	1.2387	N.S.
2. Volume of urine (ml.)	Mean 756.5	478.9	4.2013	<0.001
	S.D. 255.0	311.9		
3. Body wt. (lbs.)	Mean 154.1	148.1	1.0135	N.S.
	S.D. 22.32	26.94		
4. Age (yrs.)	Mean 21.6	28.8	4.6885	<0.001
	S.D. 3.95	8.9		
5. Sex	47 males	20 males		
	6 females	3 females		

Discussion:

Results are shown in form of histograms in Fig. 17.

The numbering of the paragraphs below corresponds to that in the Table above.

1. There was no significant difference in methacetin test result between normal and schizophrenic subjects. A single exception to this statement is treated as a special case in a later section.

2. The difference in urine volume found in the present study between samples from normal subjects and samples from schizophrenics attains high statistical significance. There are several possible

reasons for this finding:-

a) in normal subjects, bladder emptying prior to testing was deemed "desirable (but not essential)." In schizophrenics it was enforced.

b) Restriction of fluid after breakfast and before testing was probably more rigorous in the mental hospital situation. All subjects were allowed "water to quench thirst " (the words used in the test protocol).

c) Fluid consumption after the consumption of methacetin may have been greater in normal subjects, motivated to produce a respectable volume of urine than in schizophrenics not so motivated.

d) Bladder emptying at the end of the test period may have been incomplete in schizophrenic subjects. Evidence collected from the protocatechuic acid test procedure (v.i.) suggests that this was probably not an important factor.

3. The difference in body weight was in the same direction as in the protocatechuic acid test results described below, i.e. of normal subjects being heavier. This effect did not attain statistical significance.

4. The difficulty in recruiting older volunteers for methacetin testing has already been commented on.

5. Sex differences between the two groups are not significant. The small number of female volunteers (only 6 out of 53) imposed

the necessity of studying chiefly male schizophrenics so as to be able to make a valid comparison.

Summary of correlation data within the group of normal subjects.

				r	p
1.	Percentage demethylated	VS	Body Wt. (see Note 1)	- 0.255	N.S.
2.	" "	VS	Urine volume (see Note 2)	-0.374	0.01>p>0.002
3.	" "	VS	Age (see Note 3)	+0.017	N.S.

Note 1. This is a satisfactory state of affairs because with a suitable dosage schedule the two factors under consideration here should not be correlated i.e. an increase in weight should not increase, or decrease the excretion percentage.

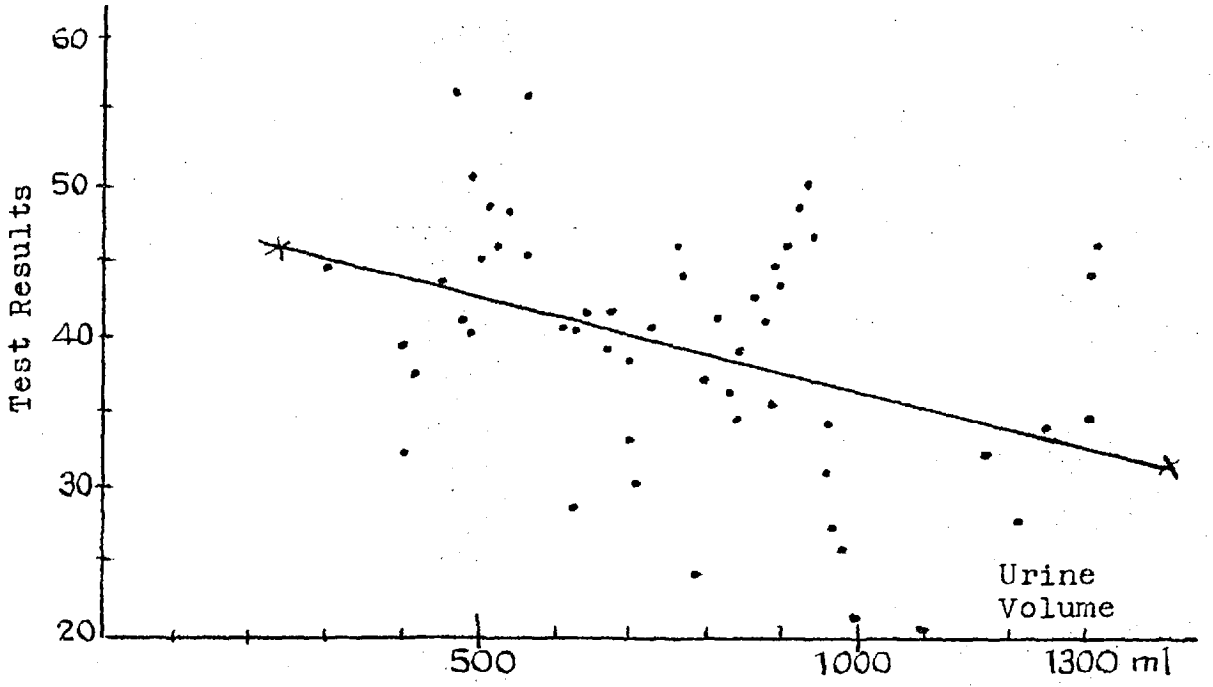
Note 2. Increased urine volume was associated with a decrease in metabolites recovered. This first appeared on graphical treatment of data. Subsequent calculations confirmed it.

If x is the volume of urine, and y the result of the methacetin test as a percentage of the dose administered, the slope b (=y/x) of the regression line is -1.184×10^{-2}; that is there is a fall in test result of 1.184 for each additional 100 cc. of urine; for this effect r, the correlation coefficient is -0.374,

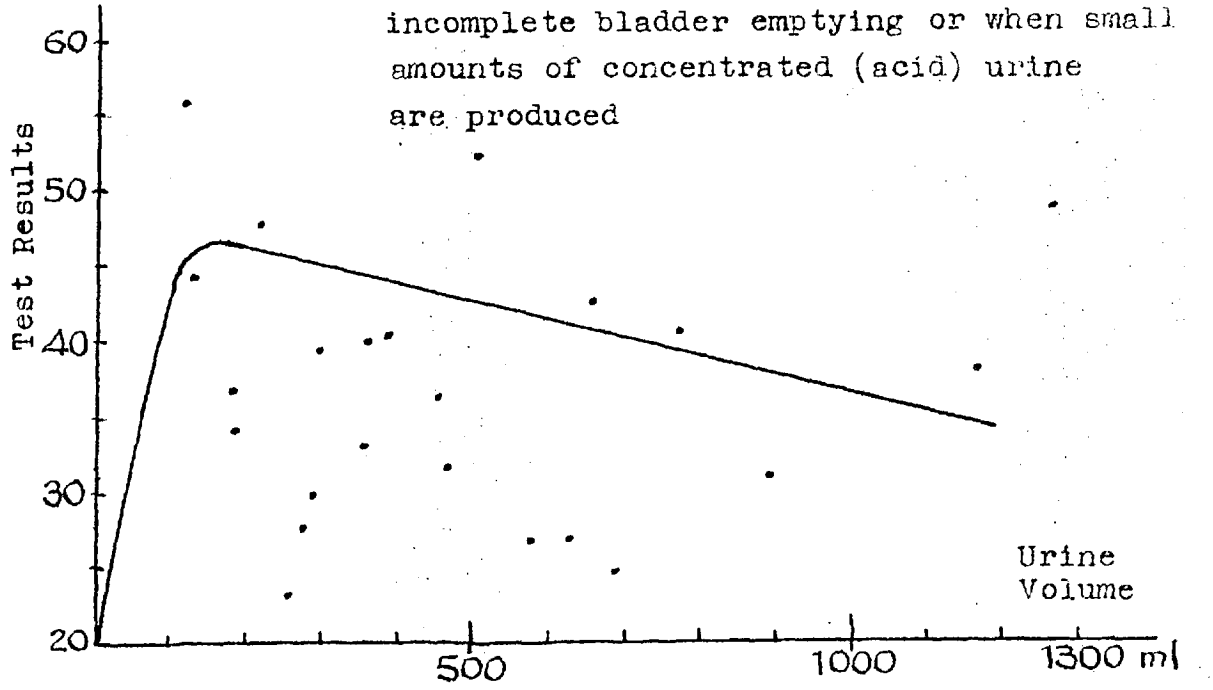
$$t = -2.861 \quad \text{and} \quad 0.01 > p > 0.002 .$$

Further analysis on the effect of urine volume on % excretion was carried out by assuming that, if x = volume in ml. and y = test

Figure 18. 53 Normal Subjects; the regression line has been drawn in.



23 Schizophrenic Subjects; the line is drawn to illustrate what may be expected with incomplete bladder emptying or when small amounts of concentrated (acid) urine are produced



result, then-

$$y = a + bx + cx^2.$$

i.e. that the relationship between x and y has a quadratic form, not a linear form; c was found not to differ significantly from zero. (This calculation was carried out using standard partial regression statistics as if x and x^2 were independent, as are x_1 and x_2 in the equation:

$$y = a + b_1x_1 + b_2x_2).$$

One can infer that this linear relationship cannot hold for all values of volume and test result. If this were to happen, zero urine volume would be associated with maximum excretion, a ridiculous result. With very low urine volumes, incomplete emptying of the bladder at the end of the test period will lead to a considerable error in test result.

For the schizophrenics in whom urine volume was significantly less than in normals, the urine volume/test correlation does not hold. Although one could correct test results for the effect of volume using the regression line method for correction, the suitability of this procedure must be open to doubt in the given circumstances.

The analytical problem posed here is illustrated in Fig. 18.

The explanation of this phenomenon can be sought by a consideration of the conditions of the test and by a consideration

of various physico-chemical factors concerned with

a) the absorption and

b) the urinary excretion of drugs.

a) Absorption effects: The conditions of the test allowed two cups of tea at about 8 a.m. , and water thereafter "to quench thirst." It may safely be assumed that the amount of fluid consumed on the day of the test before the test varied considerably from one subject to another. Those who had consumed most fluid would pass the largest quantities of urine during and after the test period. The intestinal concentration of the drug must be an important factor in drug absorption (Smyth, 1964) and in drugs diffusing through the intestinal wall, the rate of absorption is proportional to the concentration of drug in the intestine. Where the subject had reduced his fluid intake prior to the test, this concentration would be higher (and the urinary output of water likely to be lower) than when fluids had been taken freely.

b) Excretion effects : Presumably the urinary conjugates of paracetamol, i.e. the sulphuric and glucuronic acid esters, will behave as weak acids in solution. Tubular absorption of these from glomerular filtrate will be facilitated in acid urine (because ionization will be depressed : Peters, 1961; Brodie and Hogben, 1957) and in concentrated urine (because the gradient across the tubular cell membrane will be raised). A strongly

acid urine is more likely to be concentrated than dilute; therefore conditions of diuresis imposing both less acid and less concentrated urine should facilitate excretion rather than delay it.

To summarise, the observed phenomenon would seem likely to be due to an absorption effect, increased dilution of intestinal contents leading to delay in absorption (and higher urine volume).

The relationship between urine volume and test result did not hold for any of the other three groups examined (v.i.). The reason for this may lie in the fact that, within the ward situation, restriction of fluid may have been practiced prior to testing (although this was not a specific instruction in the test protocol).

Note 3. As one would have anticipated from the narrowness of the age range, there is no correlation between age and test result ($r = +0.0167$). The average age of the test subjects was 21.6 years, the standard deviation being only 3.95 years.

Summary of correlation data with the group of schizophrenic subjects.

			r	p
1.	Percentage demethylated	v.s. Body Wt.	-0.1844	N.S.
2.	" "	v.s. urine vol. (see note)	+0.0033	N.S.
3.	" "	v.s. age	+0.3794	N.S.

Note. The statistically significant inverse relationship found

between urine volume and test result found with normal subjects (and discussed in detail above) did not hold with this group of subjects.

The effects of phenothiazines on the metabolism of methacetin.

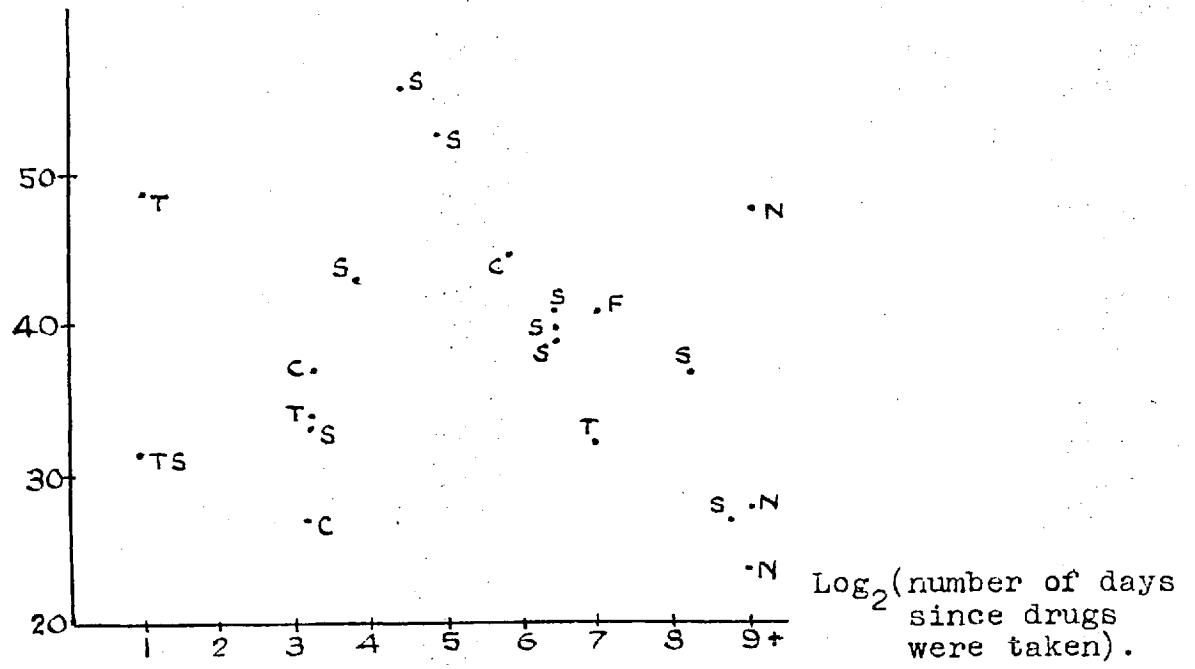
One of the difficulties with any biochemical investigation into schizophrenia is that phenothiazines (the most commonly used psychotropic agents in schizophrenia) have a multitude of effects and, furthermore, they are cleared only very slowly from the body.

Several types of effect may occur. One example is induction of enzyme activity (this occurs when chlorpromazine induces the enzyme that N-demethylates morphine in mice; Shuster and Hannam, 1965). Alternatively, inhibition of enzyme activity which may be non-competitive or competitive can take place. An example of the latter is to be found in the work of Huang and Hirano (1967) who showed that the amount of chlorpromazine which was converted into chlorpromazine glucuronide was decreased by the administration of acetanilid. The latter compound is hydroxylated in the body to paracetamol and paracetamol glucuronide is subsequently formed. The authors comment that acetanilid possesses the ability to compete with chlorpromazine in the formation of glucuronide. It may be noted that any ability that chlorpromazine has to compete with paracetamol in the formation of glucuronide may achieve extra importance for the reason that chlorpromazine, unlike some

Figure 19. Methacetin Test Result plotted against length of time off drugs.

KEY.

T = Thioridazine.
S = Trifluoperazine.(Stelazine).
C = Chlorpromazine.
F = Fluphenazine.
N = None.



other phenothiazines, is administered in a dosage of several hundred mgm./day in schizophrenia. Where a low dose phenothiazine such as trifluoperazine (daily average dose around 15 mgm.) is administered, competitive inhibition will be less important.

In order to simplify the treatment of this problem, Fig. 19 has been provided. In this the methacetin test result is plotted against length of time off drugs in days. The latter is shown on logarithmic scale in order to cope with the large spread of values. The point has already been made that the length of time off drugs must be regarded as speculative in many instances as also must the dosage at the time on discontinuation of treatment. Since different phenothiazines may exert different effects (as mentioned above in the discussion on glucuronide formation) the nature of the drug consumed is indicated by a reference letter.

Correlation coefficients derived by plotting the test result against time for the points corresponding to each drug shown on Fig. 19 did not reveal any statistically significant associations. No method of estimating tissue phenothiazine levels was considered feasible using available facilities. The method of evaluation presented here is necessarily a crude one; the problem which is both complex and difficult is best dealt with by carrying out tests on patients who had been off drugs for periods of months, and this was the policy adopted, particularly in the latter part

of the demethylation experiment, a policy which was carried over into the methylation experiments presently to be described.

The selection of chronic mental hospital patients for testing. Methocctin testing was applied to two groups of 31 chronic mental hospital patients. The first group consisted of patients diagnosed as schizophrenia, this diagnosis appearing on the case sheet and being confirmed by a psychiatrist of University lecturer status who interviewed each patient individually.

The non-schizophrenic group had diagnoses other than schizophrenia and were patients in whom the diagnosis of schizophrenia had at no time appeared on the hospital records (which were carefully perused by the author).

In both groups no patient with known non-psychiatric disease was included. All patients had been off all drugs for a minimum of seven months. A few patients had never had drugs.

Patients from both groups lived in the same hospital wards and were tested on the wards in which they lived.

Summary of Data for Chronic Schizophrenics and Non-Schizophrenic Mental Hospital Patients.

(Test result = amount of methacetin accounted for in the form of paracetamol plus paracetamol conjugates in the four hour urine collection, as a percentage of the administered dose).

		Non-Schizophrenics	Schizophrenics	Both groups combined.
Total number		31	31	62
Males		20	20	40
Females		11	11	22
Sexes considered together : Means ± S.D.				
Test result		28.6 ± 8.54	31.5 ± 7.52	
Weight (lbs)		135.5 ± 27.2	132.1 ± 23.2	
Volume (ml.)		562.9 ± 248.6	581.4 ± 272.9	
Age (yrs)		57.8 ± 10.77	57.6 ± 6.33	
Weight/test correlation	p:	N.S.	N.S.	N.S.
Volume/test	p:	N.S.	N.S.	N.S.
Age/test	p:	$0.01 > p > 0.001$	N.S.	N.S.
Sexes considered separately : Means ± S.D.				
Males : Test result		29.6 ± 9.11	33.7 ± 6.53	31.6 ± 8.09
Females: Test result		26.9 ± 7.49	27.6 ± 7.91	27.2± 7.53
For the difference between males and females	t:	0.848	2.294	2.09
	p:	N.S.	$0.05 > p > 0.02$	$0.05 > p > 0.02$
Age of males (yrs) Mean ± S.D.		57.85 ± 7.59	57.60 ± 5.94	
Age of females (yrs) Mean ± S.D.		57.82 ± 12.27	57.73 ± 7.28	

Comparison between groups (both sexes included).

In numbers and sex distribution, the groups of schizophrenics and non-schizophrenics were identical. There was no significant difference (i.e. at the 0.05 level) between groups with respect to weight, age, volume of urine or methacetin test result.

Differences between test results for schizophrenics and non-schizophrenics were not significant when data from each sex were considered separately.

Differences between the four groups studied (the two groups of chronic patients plus the group of acute schizophrenics and the group of normal subjects).

For more men were methacetin tested than women (total numbers men : 107, women : 31). Since a significant difference between test results was found between men and women in the chronic non-schizophrenic group, the statistical treatment of the four groups which follows has been applied only to men.

Designation	Number in group	Mean ± for test result	Mean age ± S.D.
1. Young controls	47	39.02 ± 7.772	21.40 ± 4.047
2. Acute schizophrenics	20	36.64 ± 8.662	29.55 ± 7.735
3. Schizophrenic mental hospital patients	20	33.67 ± 6.533	57.60 ± 5.940
4. Non-schizophrenic mental hospital patients	20	29.59 ± 9.105	57.85 ± 10.245

Using the test results, an analysis of variance for the result on the four groups yields the following:-

Source of Variation	Sum of squares	Degrees of freedom	Mean Sq.	F. ratio
Between groups	1362.53	3	454.18	7.097
Residual	6590.98	103	63.99	-
TOTAL	7953.51	106		-

With 3 and 103 degrees of freedom, the observed value of F (7.097) is significant at the 0.001 level indicating that differences between the groups are highly significant.

TABLE X A Between Groups Comparison for the Methacetin Test Results.

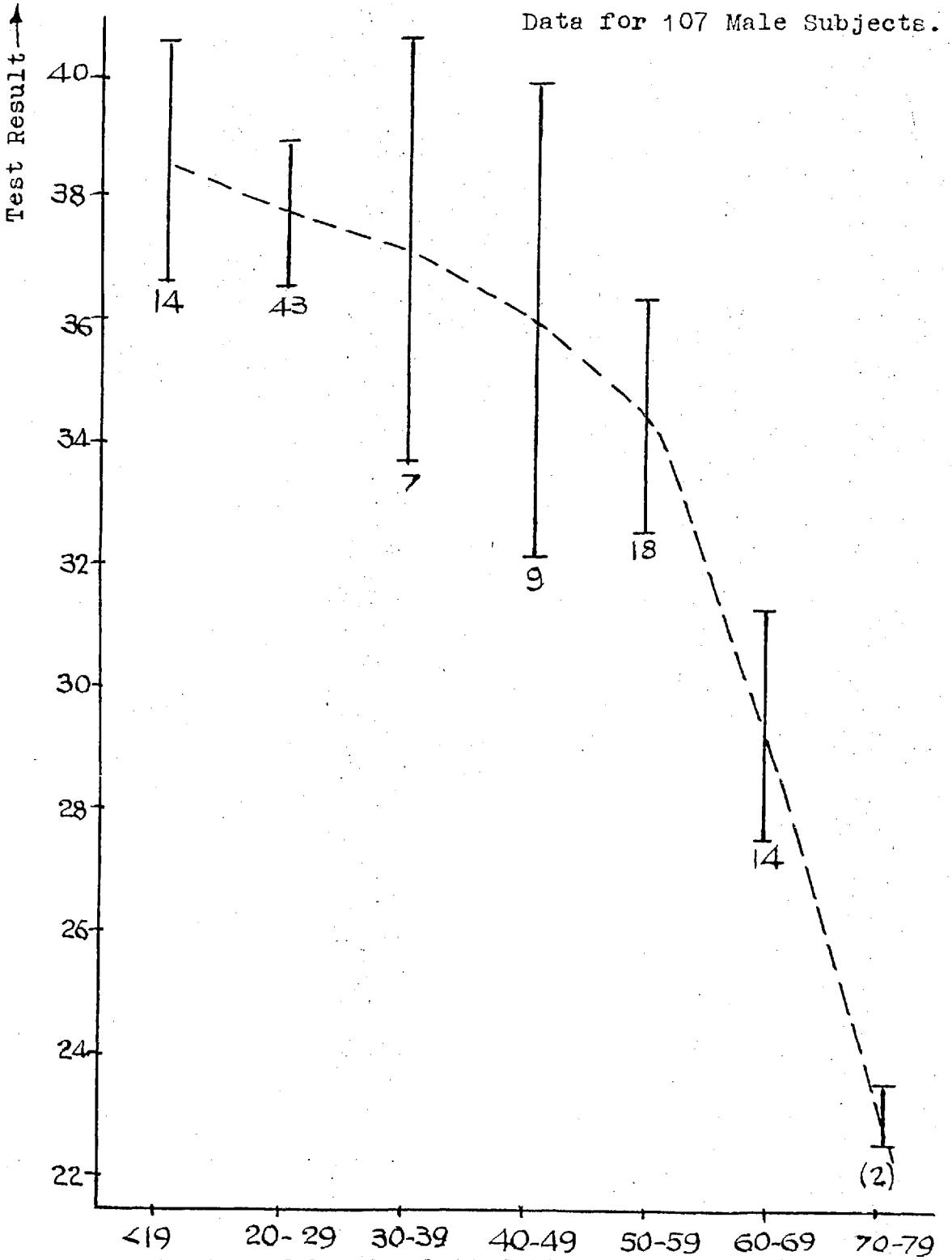
Values above the oblique line are values of Student's t; values below are values for p, the probability coefficient. The numbering of the groups is that used on the previous page.

Groups	1	2	3	4
1		1.12	2.5	4.4
2	>0.2		1.17	2.78
3	<0.02	>0.2		1.61
4	<0.001	<0.01	>0.1	

Discussion:

The prime objects were comparisons between groups 1 and 2; and between groups 3 and 4 (of methacetin test results). In both instances no significant difference has been demonstrated. The difference between groups 1 and 2 tended to favour the demethylation hypothesis, but these two groups differed significantly in age and age would appear to have an effect on test result (v.i.). In groups 3 and 4, the age difference difficulty has been largely overcome, although the standard deviation for age in the non-schizophrenic group was larger than in the schizophrenic, a fact

Figure 20. METHACETIN TEST RESULT & AGE.
Data for 107 Male Subjects.



Points joined by the dotted line are mean values for each decade. Vertical lines are ± standard error for each decade. The number under each line is the number of subjects in each group.

which probably accounts for the negative age/test correlation ($r = -0.4577$, $t = 2.772$ and $0.01 > p > 0.001$) in the former group.

Urine volume did not differ significantly between groups 3 and 4 (as was the case between groups 1 and 2), nor were test urine volume and test result related in either group 3 or group 4, making direct comparison easier. The non-schizophrenic mental hospital patients had the lower mean methacetin test result although the difference is not significant when this group is compared with the group of chronic schizophrenics.

Such differences as appear between groups are, with the exception of that between 2 and 3, statistically significant between the two youngest and the two oldest groups, suggesting an effect related to age.

That age is the responsible factor is suggested by the graphical appearance when results for all 107 male subjects are plotted by decades (Fig. 20). Although a regression line with a significant slope could doubtless be obtained for this effect, this has not been worked out, for figure 20 suggests that a linear relationship does not describe what is observed, namely a relatively rapid fall off in test result from the 7th decade (60 - 69) onwards.

The effect of age on methacetin test results is not, of course, surprising. It is in agreement with findings reviewed by Bender (1965).

A comment on the significantly lower test results found in female mental hospital patients. There is a good deal of evidence that liver microsomal enzymes (including an O-demethylating enzyme : Kato and Gillette, 1965 and an enzyme responsible for glucuronide formation : Inscoe and Axelrod, 1960) are, in the rat, more active in males than females. This sex difference is abolished by starvation. In male rats castration impairs liver microsomal enzyme activity ; in females anabolic steroids enhance it.

Kuntzman et al. (1966) give data for three human livers in the enzymic de-ethylation of phenacetin. Two livers from females had an activity of 83.3 and 67.0 units; liver from a male subject had activity of 110.0 units. This is inconclusive but suggests that the sex difference may be in the same direction in humans as in the rat and points to the need for further study.

One would expect younger subjects, with higher sex hormone levels, to demonstrate an effect due to such hormones more clearly than the more aged mental hospital patients. However, the six younger women studied had a higher mean methacetin test result than males, although this can hardly be regarded as conclusive. The explanation for the significantly lower results obtained in women may lie elsewhere than in the activity of their liver microsomes (for example in their body composition) but further studies would be required before a conclusion can be reached on

this point. For example, if the observed sex difference were abolished when paracetamol was substituted for methacetin, sex differences in activity of the O-demethylating enzyme would become more likely.

A comparison between the mean methacetin test results for the two groups of 11 chronic female patients (schizophrenic and non-schizophrenic) gave a value for t of 0.1355 ($p > 0.2$).

The methacetin test in patients ill with non-psychiatric disorders. One disadvantage of using methacetin as a test substance is that one cannot compare the amount of one metabolite excreted with that of another (see 1. Rate of methylation, p 185).

For this reason it seemed advisable to see what effect certain forms of parenchymal disease had on the test results. Patients were selected with gastrcintestinal disorders (because the test substance has to be absorbed before catabolism can start); with liver disease (because the demethylating enzyme is a liver enzyme and the enzymes responsible for the conjugation of paracetamol are partly hepatic); with renal disease (because urinary constituents were being estimated) and with myxoedema because of the general decrease in metabolism associated with this condition. Only cases where the diagnosis was not in dispute were used.

TABLE XI Methacetin test results (and other data) on patients with non-psychiatric disorders.

Age	Sex	Diagnosis	Other inform.	Vol. of urine, ml	Methacetin test
39	F	Cirrhosis of Liver	Disseminated lupus erythematosus Varices (confirmed at P.M.)	940	14.3
51	F	Cardiac cirrhosis	C.C.F.; tricuspid incompetence	410	3.0
50*	M	Cirrhosis of liver	Also hepatic secondary deposits from carcinoma of oesophagus	180	13.5
41	M	"	Haematemesis a few weeks previously. Portosystemic encephalopathy	300	9.5
26.	F	Infective hepatitis	Serum bilirubin 7.3 mgm% at time of test	100	44.6
39*	F	Primary biliary cirrhosis	Serum bilirubin 8 mgm% at time of test	270	9.0
27	M	Chronic glomerulo-nephritis	Blood urea 130 mgm%	360	8.1
25*	F	"	Blood urea 75 mgm.%	485	15.5
43	M	Malignant hypertension	Blood urea 165 mgm%	345	8.9
61	M	Prostatic hypertrophy	Blood urea 49 mgm.%	290	13.7
57	M	Pernicious anaemia	On treatment	1035	42
51*	M	Gluten induced enteropathy confirmed by jejunal biopsy	Faecal fat 8.7 G/24 hr.	105	22

TABLE XI cont/d.

Age	Sex	Diagnosis	Other inform$^{n.}$	Vol.of urine	Methacetin test
46*	F	Gluten induced enteropathy confirmed by jejunal biopsy	Faecal fat 13 G/24 hr.	370	17.5
41	F	Myxoedema	Untreated	275	24.2
41	F	"	After 6/52 treatment (L-Thyroxine 0.3 mgm. daily)	310	33.9

Note : Patients in the above Table marked with an asterisk had received treatment with barbiturates prior to testing and this, by inducing their drug metabolising microsomal enzymes may have led to higher values for the methacetin test result than would otherwise have been obtained (see Levi et al., 1968).

Discussion:

The low results obtained for patients with chronic hepatic and renal disease is shown in Table XI. The patient with infective hepatitis gave a normal result.

As far as the two patients J.N.D. and L.L., who had a biopsy based diagnosis of gluten induced enteropathy, it is worth noting the rather low values obtained (17.5 and 22%); whereas a

subject with gastric atrophy (i.e. with pernicious anaemia) had a normal test result. These findings suggest small intestinal absorption of the drug rather than major absorption through the stomach and justify insistence on a fat free breakfast as part of the test regime, this procedure being designed to prevent the delay in gastric emptying which is associated with the presence of fat in the stomach.

The abnormal methacetin test results found in a schizophrenic described in a subsequent section required exclusion in this patient of chronic renal and hepatic disease and malabsorption, any of which conditions might have given the low results obtained (v.i.).

The replicability of the test.

One important characteristic of any test is that it should be replicable.

In order to investigate this, the methacetin test was repeated on twenty male members of the mental hospital population of older patients.

Ten patients were chosen at random from each group of twenty non-schizophrenic male patients and twenty male schizophrenics (except that any patient chosen for the second test who had been put back on drugs in the interim period (average four months) was excluded from the investigation).

Initial & no. of schizophrenic		First test	Second test
1	R.McD.	29.0	29.1
2	T.M.W.	32.7	40.7
3	J.T.	29.2	32.3
5	F.W.	43.7	41.5
8	F.L.	34.4	31.6
16	C.F.	30.7	28.8
18	G.S.	30.2	23.1
19	J.C.C.	30.0	29.9
20	J.H.	32.2	25.9
26	J.G.	32.2	23.2

Initial & No. of non-schizophrenic		First test	Second test
1	B.B.	40.5	34.9
3	F.M.	46.7	46.6
4	F.S.	22.7	22.2
5	R.A.	25.4	28.7
12	J.S.	36.8	24.7
16	R.G.	28.5	31.7
17	T.U.	20.3	25.8
18	J.W.	29.2	16.0
19	W.G.	47.0	38.0
20	G.A.	21.4	35.3

Statistical Evaluation:

These data give results that can be set out as an analysis of variance, as follows:-

Source of variation	Sums of sq.	Degrees of freedom	Mean Sq.	F. ratio
Between pairs	1723.14	19	90.69	3.864
Within pairs	469.44	20	23.47	
TOTAL	2192.58	39		

For 19 and 20 degrees of freedom F is significant at the 1% level, i.e. there is significantly more variation between pairs than within pairs.

Conclusion:

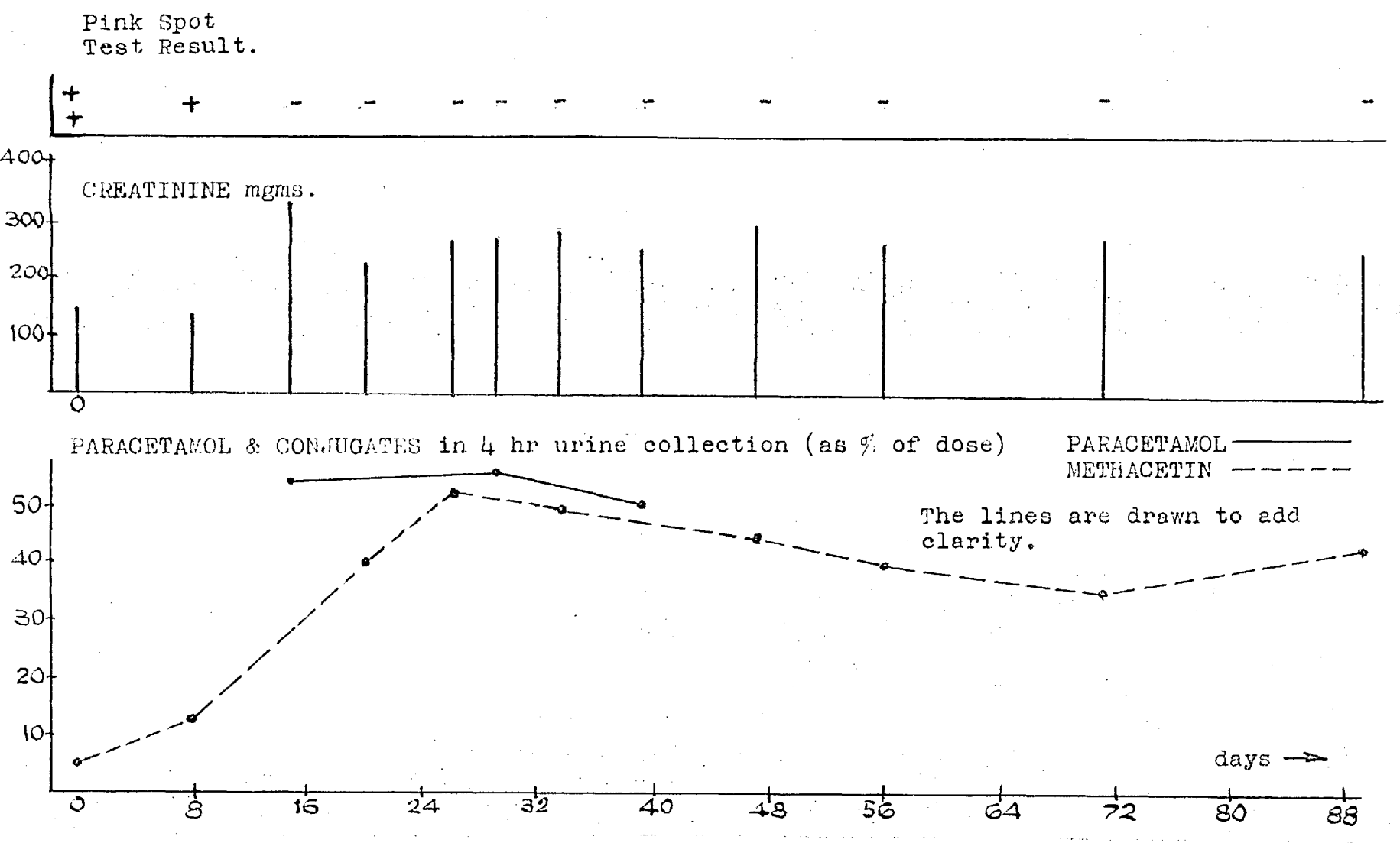
The methacotin test shows satisfactory replicability.

Difficulties encountered during methacetin testing. The difficulties experienced with acute schizophrenics and with chronic mental hospital patients, both schizophrenic and non-schizophrenic, are summarised overleaf.

Initials	Age	Sex	Diagnosis	Difficulty
J.A.	37	F	Acute schizophrenia	Patient ran into W.C. and passed urine.
J.J.	19	M	"	Urine passed after 3 hrs. but not thereafter.
O.F.	57	M	Chronic "	Urine and faeces passed together during the test
P.M.	60	F	" "	" " " " "
R.S.	38	F	" "	Urine passed after 3¼ hrs. but not thereafter.
D.M.	65	M	" "	No urine passed at all.
J.H.	60	M	Idiopathic epilepsy	Urine passed after 3¼ hrs. but not thereafter.
J.R.	60	M	Mental subnormality	Urine spilt by the patient, deliberately.
G.B.	68	F	" "	Incontinence of urine
J.C.	65	M	" "	" " "
H.W.	54	M	" "	" " "

Note: All times given refer to the time after methacetin administration. It will be noted that difficulty was never experienced in persuading the subject to consume the test substance. None of these test results has been incorporated in the calculated results.

Figure 21. RESULTS OF TESTING IN SUBJECT P.B.



A single case having unusual methacetin test results. Schizophrenic P.B.

The methacetin testing of acute schizophrenics gave results within the normal range for all patients tested, with one exception.

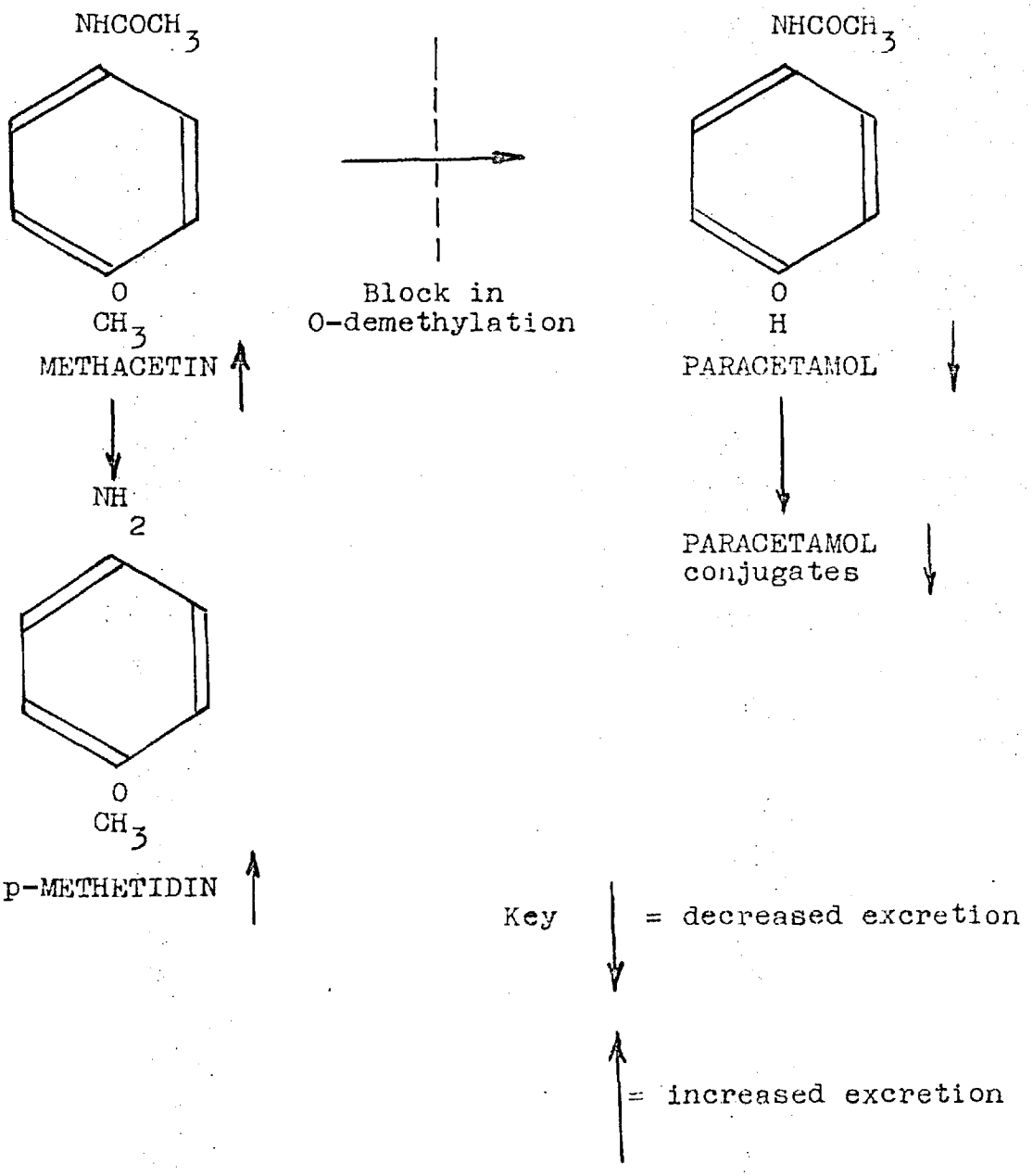
The exception was a male patient of 21, P.B., who had been diagnosed as schizophrenic from the age of 18 onwards.

He was transferred to a psychiatric unit from a general hospital to which he had been taken with mild burns to his chest which had resulted from him setting fire to his tie, and for which he received only local treatment. His manner was bizarre and he was reported as showing marked thought disorder. He denied hallucinations but felt his body and head to be out of propertion to one another; he reported that neighbours were watching him; and that the voices of people who talked to him seemed flat; he felt depressed in mood.

In the six months prior to admission to the psychiatric unit he admitted that he had taken previously prescribed phenothiazines only "now and again." He had been taking a normal diet.

From day 3 onwards the patient received chlorpromazine 150 mgm. t.d.s., Fluphenazine hydrochloride, 2 mgm. t.d.s. and benztropine methane sulphonate, 2 mgm. daily, (see Fig. 21 and Table XII, p. 168). It will be seen that the first two methacetin tests gave abnormal results (5.4% and 13.5%). As well as repeated methacetin tests, several tests using equivalent amounts of paracetamol were

Fig. 22 The changes expected in the pattern of urinary metabolites of Methacetin, if O-demethylation is blocked.



interspersed as indicated by the dates of testing.

Apart from the abnormal methacetin tests the patient was remarkable for showing very strongly the urinary pink spot phenomenon immediately before the first and second methacetin tests but not subsequently. The other noteworthy aspect of the patient has been his failure to show much clinical response to phenothiazines. He has spent almost all the time since he was first tested in hospital where his clinical state is now characterised by delusions of persecution, auditory hallucinations, motor restlessness, and blunting of affect. Insight has only been slightly maintained. Occasionally he shows outbursts of aggressive behaviour accompanied by emotional lability.

Further analysis : 1. Of Non-demethylated metabolites.

High total values for urinary methacetin and/or p-methetidin combined with low values for paracetamol and conjugates would have provided strong evidence for a block at the demethylation stage (see fig. 22).

Methacetin and p-methetidin can be estimated by the method used by Brodie and Axelrod (1949) for estimating phenacetin and p-phenetidin (see Fig. 13).

Method:

If phenacetin is extracted from alkalinized urine (pH 6.0 or greater) into benzene and the benzene is evaporated down, the

TABLE XII RESULTS ON SUBJECT P. B.

Day	Test M=Methacetin P=Paracetamol	Test result%	Unconjugated Paracetamol%	Non-demethylated metabolites%	Urine creatinine%	Urine pH	Urine volume	Pink spot test
0	M1	5.4	0.31	0.09	156	5.7	965	++
8	M2	13.5	0.6	0.2	149	5.8	1340	+
15	P1	54.4	2.9	-	325	6.1	1881	-
19	M3	40.5	2.8	0.07	219	5.4	787	-
26	M4	52.3	3.6	0.85	259	6.3	642	-
29	P2	55.8	3.9	-	265	5.8	1843	-
33	M5	50.0	2.7	0.1	289	5.9	392	-
39	P3	50.8	3.4	-	259	6.4	2090	-
47	M6	44.8	4.0	0.17	303	5.7	1195	-
57	M7	39.8	2.3	0.06	266	5.7	243	-
71	M8	36.3	4.1	0.32	274	6.2	157	-
85	M9	41.7	3.4	0.09	245	6.0	620	-

Notes 1. Wt. of subject 151 lbs. 2. Dose of methacetin = 542 mgms.

3. Equimolecular dose of paracetamol = 496 mgms.

4. % = % of administered dose in the 4 hour urine collection.
To calculate these percentages for unconjugated paracetamol, the amount present (in mgm) was multiplied by $\frac{100}{496}$

resulting solids will contain a substantial part of the phenacetin. This residue is taken up in acid and hydrolysed for 45 mins. in a water bath, the phenacetin being hydrolysed to p-phenetidin. A procedure of diazotization can then be carried out and ultimately a dye is formed which can be read in a spectrophotometer.

Such a procedure (Procedure A) carried out on urine after oral phenacetin has been consumed, estimates phenacetin and p-phenetidin. If the acid hydrolysis is omitted only the p-phenetidin is estimated (Procedure B). The phenacetin content of the specimen can then be worked out by subtracting the result from Procedure B from that of Procedure A.

This method was found to be applicable to methacetin. When 8 µG methacetin was added to 5 ml. N/2 HCl (as a suitable standard), a final spectrophotometer reading of 0.45 was obtained (using a Unicam 600 spectrophotometer).

Results:

The results obtained are included in Table XII. Values obtained in M1 and M2 were neither higher nor lower than values obtained for the other tests. (Procedure A(v.s.) used throughout).

Discussion:

This study was complicated by the observation that urine obtained just <u>before</u> the administration of methacetin gave values which were almost half as high as those of the test samples

themselves, a finding which seriously jeopardized the interpretation of results. The two pretest specimens available for testing, specimens collected prior to M3 and M4, were obtained while the patient was on drug therapy (as indeed were all pretest specimens except for the first), but urine obtained from a normal subject who had consumed no drugs for several months gave a similarly high blank reading.

All figures given for non-demethylated metabolites (below and in Table XII) are uncorrected for blank values.

In the first test, M1, (carried out prior to the start of therapy) the total amount of non-demethylated metabolites was 0.09% of the dose; values were similarly derived for five other schizophrenics (patients 8, 13, 14, 16 and 22). These samples were found to contain 0.09, 0.07, 0.07, 0.15 and 0.06% of the administered dose respectively; the result for P.B. fell within this range of values. (The very low values obtained throughout presumably reflect the efficiency with which the renal tubules reabsorb the relatively lipid soluble compounds methacetin and p-methetidin).

To summarise, the rather unsatisfactory estimations of non-demethylated metabolites did not provide support for the existence of a block in the demethylation of methacetin in the patient P.B. at the time of tests M1 and M2.

Further analysis : 2. Of paracetamol conjugation.

By the time Cummings et al. (1967) had developed a T.L.C.

method for separating paracetamol, paracetamol sulphate and paracetamol glucuronide, insufficient urine from tests M1 and M2 was available for analysis using their method.

Cummings et al. found that, after an oral dose of paracetamol, four subjects excreted in the subsequent $4\frac{1}{2}$ hours, the amount of the three metabolites that are shown in Table XIIa , subjects 1 - 4, the figures referring to the percentage of each metabolite relative to the total excretion (p = free paracetamol, ps = paracetamol sulphate and pg = paracetamol glucuronide).

This Table also shows the amount of unconjugated paracetamol appearing in the urine of P.C. No increase in unconjugated paracetamol is noted for tests M1 and M2 as might have occurred had the conjugation of paracetamol been faulty.

TABLE XIIa Excretion of free paracetamol in 4 subjects (1 - 4) (as noted by Cummings et al. (1967)) and by P.B., as % of total paracetamol excretion.

Subject	p	ps	pg	Total
1	7	39	54	100
2	6	36	58	100
3	6	39	55	100
4	7	38	55	100
M1	5.7	Not	done	100
M2	4.4	"	"	100
P1	5.3	"	"	100
M3	6.9	"	"	100
M4	6.9	"	"	100
P2	7.0	"	"	100
M5	5.4	"	"	100
P3	6.7	"	"	100
M6	8.9	"	"	100
M7	5.8	"	"	100
M8	11.6	"	"	100
M9	8.2	"	"	100

Note : Free paracetamol was estimated by the same method that was used for total paracetamol but without preliminary acid hydrolysis. In order to obtain Unicam readings that were not inconveniently low, 5 to 10 ml. urine was used for the analysis.

General discussion of the problem presented by patient P.B. It is evident that the results of test M1 and M2 are very low. This phenomenon requires explanation.

If the explanation lies elsewhere than in a failure of demethylation, then the most likely explanation is that the test procedures were incorrectly carried out. The second test was supervised by a responsible senior member of the nursing staff, and there seems little reason to suppose that it was incorrectly carried out. Yet the creatinine value for this test was lower than that of any other test on P.B. This creatinine value (149mgm/4 hr) was far lower than that of any of 55 control subjects discussed later (see p 277) although one schizophrenic produced a much lower value.

Urinary creatinine has been studied more systematically in a study presented later in this thesis, that concerned with the methylation of protocatechuic acid. Evidence will subsequently be presented for the view that urinary creatinine levels may be related to the clinical state of the patient, the agitated, apprehensive subject being more likely to have higher values than normal for urinary creatinine, the anergic withdrawn patient lower values than normal. On clinical grounds the patient P.B. would fit better into the latter than into the former category.

On this evidence it does not seem logical to discard M1 as being

invalid although it must be admitted that it was carried out in the usual way without any special observation being applied as was the case with M2. The 'usual way' means that, although the test substance was administered by a senior member of staff, the patient was then kept for the remainder of the test period in a small observation ward having about 10 beds, where he was watched by attendants and, when he wished to pass urine, escorted to a side room (but not left there alone).

It will be noted that the volumes of M1 and M2 are large so that errors occuring through incomplete voiding of urine at the end of the 4 hr. test period should not do more than have a slight influence on the test result. For both tests M1 and M2 it is difficult to see how loss of urine during the test period could have occurred, nevertheless this possibility cannot be entirely excluded.

There was, in this patient, no clinical evidence of malabsorption; nor was there any clinical or biochemical evidence whatsoever of malabsorption, liver dysfunction or renal disease, such as might have accounted for the low results obtained for M1 and M2. Had the patient had such disorders, subsequent normal results would have been difficult to explain, unless P.B. had liver disease and phenothiazine therapy had induced liver microsomal activity.

Possible explantations of low test results M1 and M2. If one accepts the possibility that a temporary fault in the demethylation system may have occurred in this patient, what explanation might be offered?

1. That the fault occurred as part of an inborn error of metabolism, and that subsequent improvement was due to phenothiazines causing an enzyme induction effect similar to that which has been described by Shuster and Hannam (1965) for the enzyme (a different enzyme) which causes the N-demethylation of codeine in mice. Unfortunately the patient's condition has not improved to a state in which the clinician responsible for his care considers that phenothiazines might reasonably be discontinued, so that this explanation cannot be readily substantiated. It may be noted that both P.B.'s parents gave normal methacetin tests, although this would be expected if one was dealing here with a recessively inherited abnormality in demethylation.

In this connection may be mentioned a report by Shahidi (1967) of a patient thought to be showing evidence of a partial failure to de-ethylate phenacetin, who presented with methaemoglobinaemia and haemolytic anaemia resulting from phenacetin consumption. She excreted only 30% of the total dose of phenacetin as paracetamol (free and conjugated), compared with a normal excretion of over 70%. A 38 year old sister showed a similar abnormality but other members of the family showed no abnormality

(this suggesting the possibility of a recessive mode of inheritance). This patient excreted a variety of 2-hydroxy derivatives - 2-hydroxyphenetidin glucuronide, 2-hydroxyphenetidin sulphate and 2-hydroxyphenacetin glucuronide. When her urine was subjected to acid hydrolysis, a highly oxidizable compound, hydroxyphenetidin was formed, which, if oxygen was not excluded, turned dark within minutes. No such phenomenon was noted when urine from the present subject was acid hydrolysed. It is difficult to judge from Shahidi's data whether inadequate de-ethylation or aberrant 2-hydroxylation should be deemed the primary metabolic error. If the latter were responsible then the explanation of abnormal methacetin test results in the present patient is likely to be due to a different mechanism. Thus, in Shahidi's patient the abnormality appeared to be permanent and it was made worse by a drug inducing enzyme (phenobarbital). In the present case the abnormality was only temporary and therapy (phenothiazine medication) was associated with improvement in the methacetin test results not deterioration (although this statement does not imply a claim for cause and effect).

2. That a temporary fault occurred in the demethylating system due to the presence of amounts of some other substance in amounts large enough to cause enzyme inhibition by competition. This seems very unlikely. The capacity of this enzyme system

is considerable and the amounts of any competing substance would have to be very large, particularly as the system has a high affinity for methacetin among a variety of substrates studied (Axelrod, 1956).

3. That there was a deficiency of some co-factor of a degree sufficient to impair the functioning of the enzyme. The most likely factor of those known (N.A.D.P. and nicotinamide) would be nicotinamide which is a consituent of N.A.D.P. (otherwise designated as T.P.N.). There was no clinical evidence of nicotinamide deficiency (although it seems doubtful whether pure nicotinamide deficiency would produce any florid clinical signs) nor was the diet abnormal before testing. Nevertheless, there is independent evidence of abnormality in nicotinamide metabolism in schizophrenia and this possibility appears worthy of further consideration.

Abnormalities of nicotinamide metabolism in schizophrenia.

In the human, a small part of administered nicotinamide is excreted unchanged; a substantial portion is excreted as N_1-methyl nicotinamide (N.M.N.), the latter yielding an excreted oxidation product, N_1-methyl-2-pyridone-5-carboxamide (2 P.Y.).

Heyman and Merlis (1963) reported that there was no significant difference after an oral dose of 1 G. nicotinamide in the total amount of nicotinamide excreted in the methylated form between normals and schizophrenics. In schizophrenics there was a significantly lower excretion of N.M.N. but a significantly higher excretion of 2 P.Y. It must be noted that the excretion of N.M.N. and 2 P.Y. was expressed as a ratio, using creatinine excretion (C) as the denominator, an undesirable manoeuvre, since creatinine excretion would seem to be subject to more variation in schizophrenics than in normal subjects.

Prolonged daily administration of nicotinamide did not alter the two ratios, (i.e. the ratios $\frac{\text{N.M.N.}}{\text{C}}$ and $\frac{\text{2 P.Y.}}{\text{C}}$) suggesting that it did not critically deplete the methyl stores.

Tranquillizer therapy led to a significant increase in the former ratio, whereas psychotomimetic drugs lead to an increase in the $\frac{\text{2 P.Y.}}{\text{C}}$ ratio.

Brown et al. (1960) compared the excretion of 2 P.Y. in eight normal and nine schizophrenic subjects (the latter being young, newly admitted patients who were therefore presumably acutely disturbed), before and after a dose of L-tryptophan. This amino-acid is converted in the body into nicotinamide via a number of intermediaries. They found a significant difference before tryptophan administration with the schizophrenics excreting less

2 P.Y. than the normal subjects did. This difference was not significant after tryptophan had been administered.

Price et al. (1959) in a rather similar study, also found less 2 P.Y. in the urine prior to tryptophan in schizophrenics, but found that the difference between schizophrenics remained statistically significant after tryptophan administration. Some of the 19 schizophrenics studied by Price were in their first psychotic episode; others were more chronic.

The results of the studies cited are summarised in Table XIIb.

TABLE XIIb Summary of data on nicotinamide metabolites in studies where normal and schizophrenic subjects have been compared.

Study	Administered agent	N.M.N.	2 P.Y.	N.M.N.+ 2 P.Y.
Heyman & Merlis (1963)	Nicotinamide	*↓	*↑	*N.S.
Brown et al. (1960)	Nil	0	↓	0
" " " "	Tryptophan	0	N.S.	0
Price et al. (1959)	Nil	0	↓	0
" " "	Tryptophan	0	↓	0

↓ = significantly less excreted by schizophrenics

↑ = significantly more excreted by schizophrenics

0 = not done

* = estimated as a ratio with urinary creatinine as denominator.

The only point of agreement in this Table is the lower values for 2 P.Y.(before tryptophan was administered),in schizophrenics than in normal subjects.

Buscaino et al. (1966) have reported that, if 'de-proteinized blood' (this presumably means defibrinated blood), betaine (a methyl donor) and nicotinamide (a methyl acceptor) are incubated together, then there is a much higher production of N.M.N. in schizophrenics than in normal subjects.

In schizophrenics they found that N.M.N. content was increased by 30% or more (compared with pre-incubation values) but in normal subjects and patients mentally ill with disorders other than schizophrenia the increase was less than 20%. In a study carried out blind, (the biochemical studies being done in ignorance of psychiatric diagnosis) in 95 subjects, the following results were obtained:-

Test +ve	: Increase of N.M.N. 30% or more	45
Test doubtful	: Increase of N.M.N. less than 20%	9
Test -ve	: Increase of N.M.N. less than 20%	41

	Acute or reactivated schizophrenia off treatment	Other diagnoses
Test +ve (45)	40	5
Test doubtful	3	6
Test -ve (41)	0	41

The test would appear on this evidence to show a clear cut difference between schizophrenics and non-schizophrenic subjects.

The methylation of nicotinamide can be carried out by a liver enzyme (nicotinamide methyltransferase) in many species (Cantoni, 1951). The methyl donor for this enzyme must be either methionine or a betaine/homocysteine combination. Whether it is the activity of this enzyme (in serum) that was being measured by Buscaino and his colleagues seems very doubtful, since they were able to use betaine as the methyl donor.

If this reaction were taking place at an unusually rapid rate in schizophrenics one would expect a <u>tendency</u> towards

a) An increase in plasma and urinary N.M.N. + 2 P.Y. provided nicotinamide (or its precursor, tryptophan) was readily available.

   Table XIIb shows no evidence of such a tendency.

b) Lower tissue levels of nicotinamide if methyl donors were readily available.

Perhaps this could lead ultimately to nicotinamide depletion and be responsible for the low values shown for 2 P.Y. in Table XIIb.

c) A mopping up of methyl donors when large doses of nicotinamide are administered with a considerable rise in N.M.N. and 2 P.Y. production. The work of Heyman and Merlis (1963) which has been quoted above does not show such a rise.

Whether the abnormality that Buscaino et al. have described could operate at a rate sufficient to convert these 'tendencies' into important reactions seems uncertain; in any event, further independant studies are required.

Whether the test results M1 and M2 on subject P.B. could relate to effect (b) in the above paragraph would also seem uncertain. Unfortunately, insufficient urine remained after the other analyses carried out on this patient had been completed for nicotinamide metabolites to be estimated.

The administration of nicotinamide to P.B. Because of Hoffer's reports of successful treatment of schizophrenics with nicotinamide, the patient was transferred from the more chronic unit to an acute unit some 28 months after he was first tested. Without altering his psychotropic drugs he was assessed at weekly intervals by a psychiatrist familiar with the use of rating scales. It was intended that this psychiatrist should be kept in ignorance of the therapy planned but a member of the staff of the acute unit "let the cat out of the bag." After three weekly ratings 3.0 G. of nicotinamide (1 gelatin capsule containing 0.75 G. nicotinamide q.d.s.) were given daily for five weeks. After three weeks on this treatment weekly rating was recommenced and continued for five weeks; three weeks after the recommencement of rating, dummy capsules (containing sorbitol) were substituted for the nicotinamide,

this substitution being disclosed neither to the ward staff nor to the psychiatrist who was rating the patient.

The results of this therapeutic experiment were entirely negative. There was no change in the patient's condition on any rating scale during or after the period of nicotinamide administration.

Hoffer himself does not claim that nicotinamide is of immediate benefit in the chronic type of patient, which the patient described here had become. He suggests that at least six months' therapy should be administered to such patients and that they then may show some improvement. The shorter period of nicotinamide administration given here was undertaken lest the patient had some special disturbance of nicotinamide metabolism (or utilisation) which might have responded quickly to massive amounts of nicotinamide.

The normal results obtained with patient P.B. on subsequent study.

It will be noted from Table XII and Fig. 21 that a considerable number of normal results of methacetin testing were obtained (M3 - M9).

It will be recalled that phenothiazine therapy was instituted on the day after the first test, and to the change to normality in methacetin test result the work of Salvador and Burton (1965) may be relevant. They studied the inhibition of the methylation

of nicotinamide by chlorpromazine, in vitro. Their findings suggest "that the administration in vivo of chlorpromazine would be expected to prolong the period that nicotinamide serves as a precursor for N.A.D. synthesis by inhibiting nicotinamide methylating enzymes." If, as has been speculated above, there was a temporary disturbance in P.B. due to his inability for any reason to utilise nicotinamide, then this might link up with the excessive methylation of nicotinamide described in schizophrenics by Buscaino and his colleagues. Chlorpromazine, by inhibiting this reaction, could allow nicotinamide to become available for other roles.

It must be emphasised that all this is highly speculative. Rather than provide any stimulus for a repetition of the work presented on methacetin, it should perhaps indicate the need for an independant assessment of Buscaino's work in an attempt to confirm the results of his blind survey.

EXPERIMENTAL SECTION 3 : METHYLATION IN VIVO.

INTRODUCTION.

The purpose of the work presented here was to investigate, using pharmacogenetic technique, the methylation, in vivo, of a suitable substrate on a quantitative basis, and to compare results in schizophrenics and normal subjects.

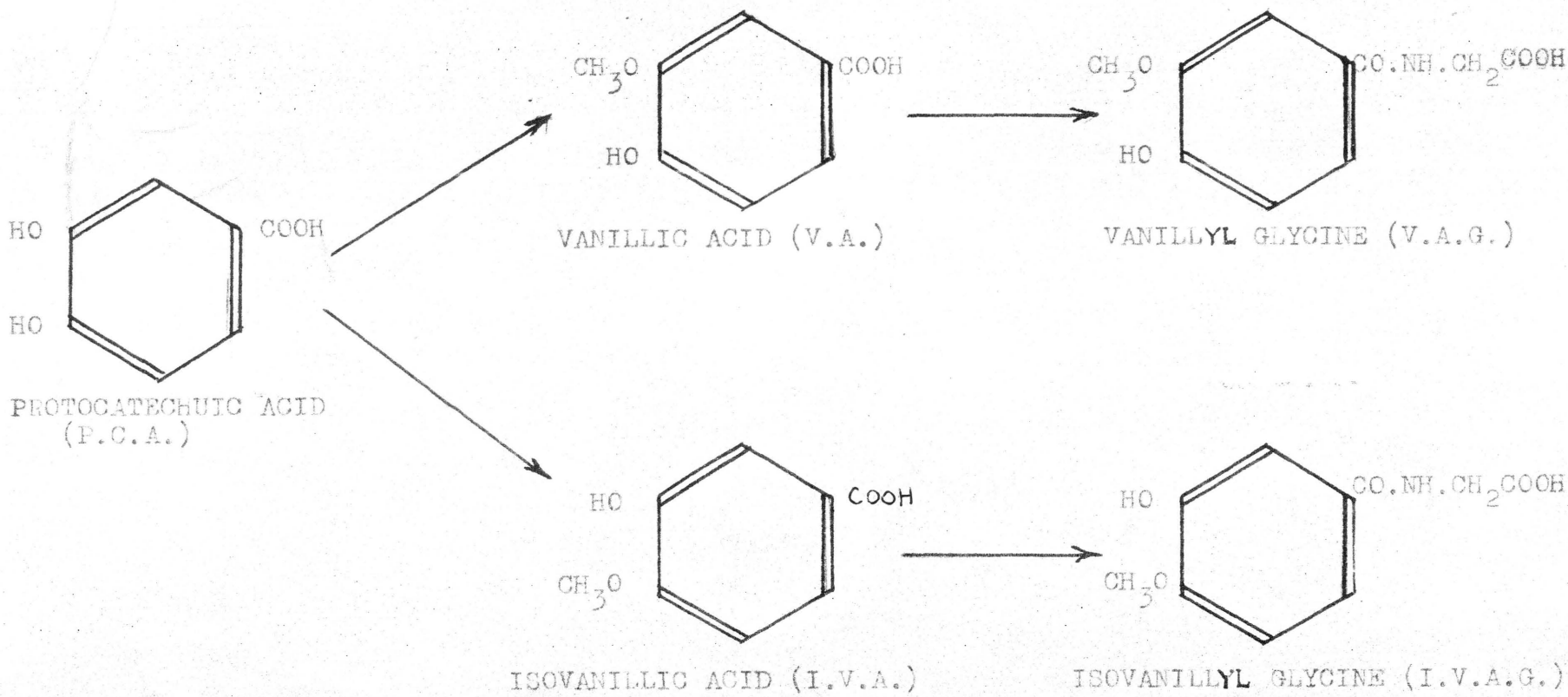
Information on two independant points was required.

1. Whether there existed any difference in the rate of methylation between a normal group of subjects and any of a group of schizophrenic subjects.
2. Whether there existed any evidence of "abnormal" methylation either in a normal group of subjects or in any of a group of schizophrenic subjects.

Attention has already been drawn to the fact that it is the 4-methoxy group which has been of most interest to psychopharmacologists, and an account has already been given of studies on the demethylation of a 4-methoxy compound in man. 4-methylation of catechols has been subjected to much less study than has the much documented process of 3-methylation. It was the measurement of 4-O-methylation which formed one of the objectives in the present study. Using the chosen substrate (v.i.) it will be shown that this process is not "abnormal," occurring in normal subject and schizophrenics alike, but (in both groups) at a much

Figure 24 The Metabolism of Protocatechuic Acid in Man.

All five substances depicted appear in the urine.
Abbreviations used in the text are given in brackets.



slower rate than does 3-O-methylation.

The substance which was used as a substrate for the methylation studies presented here is a 3:4 dihydroxybenzoic acid (protocatechuic acid, PCA). The advantages and disadvantages of using this substance are considered in a later section (see p 285). As an introduction to the metabolism of PCA reference may be made to Fig. 24, which also gives the letters used as abbreviations for the compounds shown.

1. Rate of methylation. In order to provide an index of the rate of methylation, a comparison has been made between the total excretion of the chief methylated metabolite, vanillic acid (total = free and conjugated), and of unaltered PCA in the urine. This is referred to as $\frac{VA(total)}{PCA}$, a convenient shorthand for the more cumbersome expression 100 x $\frac{\text{amount of free and conjugated VA excreted}}{\text{amount of parent compound excreted}}$. (As an alternative the ratio $\frac{\text{amount of free and conjugated VA excreted}}{\text{dose of PCA}}$ could have been used, but it seemed likely that the latter might be more influenced than the former by variability in absorption and excretion, such variability being irrelevant to the objectives of the study.) Excessively rapid methylation would result in a high value for this ratio.

2. 4-O-methylation. After effective acid hydrolysis, a urine specimen containing PCA and its metabolites will no longer contain

glycine conjugates, the methylated metabolites present being simply in the form of vanillic acid and isovanillic acid. The ratio of the amounts of these that are present will be referred to as the $\frac{VA}{IVA}$ ratio. This, more fully is -

$$\frac{\text{vanillylglycine} + \text{vanillic acid}}{\text{isovanillylglycine} + \text{isovanillic acid}}$$

The $\frac{VA}{IVA}$ ratio therefore represents the relative total amounts of 3-methylated to 4-methylated metabolites excreted. Excessive 4-methylation would result in a low value for this ratio.

A route for the formation of false neurotransmitters in man.

In order to justify a test such as the protocatechuic acid test (q.v.), which is making an overall measurement of a) the rate of methylation and b) the type of methylation (i.e. whether 3-O or 4-O), it must be shown that pathways exist such that an examination of the transformations being measured could be relevant to the problem of schizophrenia.

Such pathways do exist and are made use of therapeutically. Thus, the drug α methyl D.o.P.A. (Aldomet) used in the treatment of hypertension, acts as follows : it is decarboxylated by D.o.P.A. decarboxylase to α methyl dopamine and subsequently hydroxylated by dopamine β hydroxylase to α methyl noradrenaline. This latter compound is then able to act as a false neurotransmitter, blocking the peripheral actions of noradrenaline; this is not a unique

effect, for it occurs with α-methyltyrosine and other similar compounds (Kopin, 1968). Calne et al. (1969) and others have recently provided evidence that D.O.P.A. may be of value in Parkinsonism. It is presumed that D.O.P.A. is converted to dopamine in order to exert an effect and it is well known that there is a deficiency of dopamine in the basal ganglia in Parkinson's disease.

What is being suggested is that the abnormal methylation of D.O.P.A. (or some other amino acid similar to D.O.P.A.) might occur and that this acid might pass the blood-brain barrier (which is more permeable to amino acids than to amines) as such; and that the normal activity of intraneuronal aromatic amino-acid decarboxylase might produce an amine which could act as a false neurotransmitter just as such a transmitter derives from α-methyl dopa.

An examination of the metabolism of D.O.P.A. itself has the disadvantage of complexity : degradation involves the interaction of a number of enzymes. Decarboxylation, transamination, O-methylation in the 3- or 4-position and amine oxidation all occur as important transformations, and other minor transformations seem very probable (Smith, 1967).

An overall increase in the rate at which biological methylation is carried out in the body could alter circulating aminoacids and provide a more methylated substrate from which

methylated amines could subsequently derive. It is the possibility of such an overall increase in methylation which the PCA test sets out to examine.

Quite apart from this, the enzymes controlling methylation in all its aspects may be the same in brain as in other organs, enzyme structure being under the same genetic control. In this situation brain enzyme abnormality would be reflected in a general abnormality such as a pharmacogenetic test could demonstrate. Thus, as far as C...M.T. itself is concerned, Axelrod (1966) in a review of the properties of this enzyme does not put forward any evidence to suggest that brain C...M.T. differs in its properties from C...M.T. in other organs. The possibility that a considerably more complex situation exists than has been thought hitherto is raised by the observation by Anderson and D'Iorio (1968) that C.O.M.T. shows multiple bands of activity after being subjected to polyacrylamide gel electrophoresis.

A note on the pharmacology of PCA. Because of the beneficial effect of 2-hydroxybenzoic (salicylic)acid as a treatment for rheumatic fever, Clarke et al. (1958) gave PCA a trial in that condition. PCA was given in a dosage of 5.3 to 6 G. per day to three patients with recurrent rheumatic fever, two of whom were children. The treatment resulted in elevation of temperature and heart rate, increased rheumatic pains and general discomfort; the

two children were reported as becoming drowsy, physically inactive, mentally depressed and they complained of headache. Sedimentation rates showed slightly increased values. The treatment was discontinued after an average of seven days. No persisting toxic effects were noted.

Clarke et al. showed that a single dose of PCA as high as 0.3 G/Kg. body weight given to rats did not produce any toxic manifestations.

On the basis of these results, it seemed reasonable to suppose that a single dose of about 1.0 G. of PCA would not produce any severe toxic effects in adult human subjects and subsequently this supposition proved to be correct : no side effects were reported.

In connection with the mental side effects of PCA it may be mentioned that Ross and Wootton (1964) found PCA to be capable of inhibiting three brain enzymes (which they studied in vitro using rat brain homogenates) : the enzymes responsible for succinate oxidation and anaerobic glycolysis, and also the enzyme glutamic acid decarboxylase. Vanillic acid exerted less inhibition on these enzymes than did PCA, while vanillylglycine was virtually inactive indicating that, as far as these brain enzymes were concerned, methylation and glycine conjugation could be regarded as detoxication processes.

A COMPARATIVE STUDY OF THE METABOLISM OF PROTOCATECHUIC ACID IN NORMAL AND SCHIZOPHRENIC SUBJECTS.

Materials.

Protocatechuic acid (PCA) was obtained from Sigma Chemical Company, being used without further purification. The compound supplied was an off-white powder which never yielded more than a single spot on any chromatographic system on which it was examined.

Vanillic acid and 3:4 dimethoxybenzoic acid (veratric acid), were obtained from the Koch-Light Chemical Company. Isovanillic acid was prepared from isovanillin and the product purified and identified as pure by the techniques described by Price (1969).

Methods.

1. The identification of the urinary metabolites of PCA. It was anticipated when the project commenced that the following might be expected to appear in the urine after an oral dose of PCA.

1. PCA itself.
2. PCA glucuronide.
3. Vanillic acid (VA).
4. Vanillylglycine (VAG).
5. Isovanillic acid (IVA).
6. Isovanillylglycine (IVAG).

These expectations derive from several earlier studies. De Eds et al. (1957) found PCA and VA in the urine of rabbits and rats

TABLE XIII Summary of methods used in comparing the authentic compounds with presumed PCA, VA and IVA.

METHOD	PCA	VA	IVA
1. Paper chromatography (ascending, Whatman No. 1).			
a) Chloroform, glacial acetic acid, water; 2 : 1 : 1 (lower organic phase).	Yes	Yes	(see note 4)
b) Isopropanol, 880 ammonia, water; 20 : 1 : 2.	Yes	Yes	(see note 5)
c) Sodium formate 10 G, formic acid 1 ml., water to 200 ml.	Yes	Yes	(see note 4)
d) 20% aqueous potassium chloride	Yes	Yes	(see note 4)
e) n-Butanol, 880 ammonia, water; 8 : 1 : 1.	Yes	Yes	(see note 4)
2. Thin layer chromatography (Silica Gel)			
a) Benzene, glacial acetic acid, water; 2 : 2 : 1 (upper organic phase).	Yes	Yes	Yes
1c) As above	Yes	Yes	Yes
3. Staining with			
a) Folin and Ciocalteu's phenol reagent (system 2 (a) only)	Blue	Blue	Blue
b) Sulphanilic acid reagent : All systems	Brown	Orange	Orange
4. U.V. spectrum of material eluted from T.L.C. plates run on System 2 (a)	Yes	Yes	Yes
Spectral shift on the addition of 1% methanolic $AlCl_3$ (Nakagawa, 1964)	Yes	Yes	Yes

Notes : 1. Yes = Same Rf or spectrum in authentic and presumed compounds.
2. Authentic and presumed compounds gave the same colours (3a and 3b).
3. No case of disagreement was found.
4. VA obscured IVA on systems 1 (a), 1 (b) and 1 (d)
5. IVA resolved poorly from VA on systems 1 (b) and 1 (e).

after administration of PCA. Booth et al. (1957) found both VA and VAG in urine after an oral dose of 1 G. of caffeic acid (3:4 dihydroxycinnamic acid) to human volunteers; the quantity of VA was estimated as being 10 mgm. and of VAG 150 mgm. Hill et al. (1959) found that methylation of PCA occurred at both hydroxy groups in humans (i.e. that both VA and IVA were formed).

The occurrence of metabolite 2, protocatechuic acid glucuronide is considered separately in a subsequent section, where it is suggested that the evidence points to it being at most a minor metabolite of PCA (see p196).

The methods of identification of PCA, VA and IVA used in the present study. Table XIII sets out the data on which the identification of the compounds PCA, VA and IVA was made in the present study.

The usual finding when samples of urine containing PCA and its metabolites were acid hydrolysed, extracted, the ethyl acetate extract chromatographed and the chromatogram stained with sulphanilic acid was of two large spots not present when pretest urine was similarly treated. One spot stained light brown; (as authentic PCA does); the other orange, (as authentic VA does). (See Fig. 26, p. 202).

On a typical paper or plate the following spots would be applied:-

1. Authentic VA.
2. Test extract.
3. Test extract + VA.
4. Pretest extract.

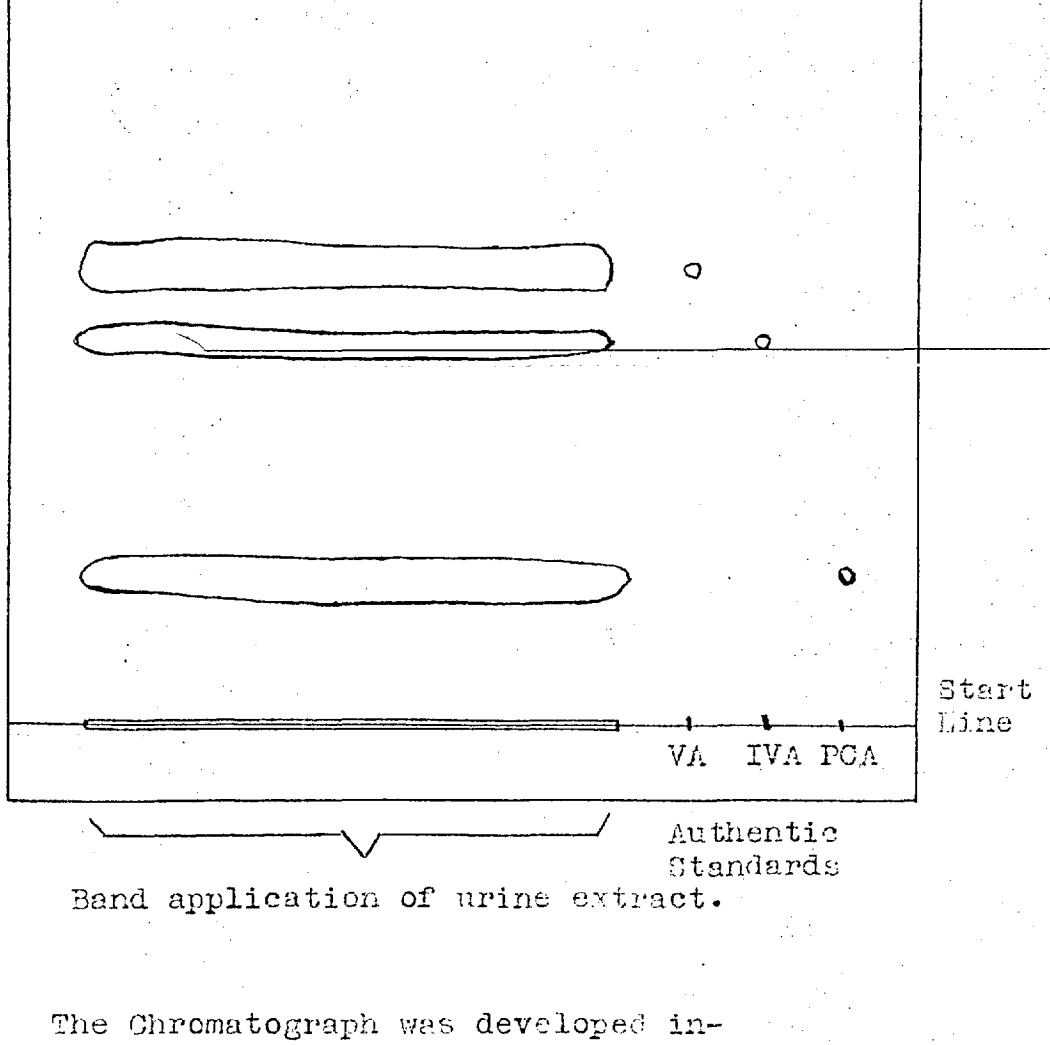
One could then show that the test extract yielded a large spot which had the same Rf as authentic VA; that adding VA to the test extract increased the size of this spot; and that a pretest extract derived from the same subject contained no such spot. This was done for each compound in each system, as shown in Table XIII by the word 'yes.'

Many solvent systems failed to achieve chromatographic separation of VA and IVA. Two systems which succeeded were benzene:glacial acetic acid:water :: 2 : 2 : 1 upper (organic) phase, (Hedin et al., 1967) and n-butanol : 880 ammonia : water :: 8 : 1 : 1 ; (Hill et al., 1959).

When a series of extracts obtained from urine collected after PCA administration and subsequently hydrolysed were compared chromatographically with a series obtained before PCA administration using either of the above solvent systems,3 chromatographic spots were found in the former which did not appear in the latter. Two of these were large spots and could be shown to be produced by PCA and VA. The third (which was much smaller) had the same Rf as authentic IVA on both systems; both authentic and presumed

Figure 25

Thin-layer plate showing the appearance after a urine 'test extract' had been chromatographed against authentic standards



The Chromatograph was developed in-
benzene : glacial acetic acid : water :: 2 : 2 : 1.

IVA stained orange with the sulphanilic acid reagent.

Spectral studies. In order to obtain sufficient presumed IVA for spectral studies, a continuous band of ethyl acetate extract (prepared from urine collected after PCA administration, which had been acid hydrolysed) was applied along a line parallel with and 1 cm. in from the edge of a T.L.C. plate incorporating a fluorophor activated by 254 mμ light.

Authentic PCA, VA and IVA were applied as markers a short distance away from each end of the band.

The plate was developed in the benzene/acetic acid/water system. Three dark bands were found opposite the three markers when the plate was examined under a 254 mμ light source (see Fig.25) The three bands were scraped off separately and the scrapings weighed and transferred into three centrifuge tubes. Three ml. of methanol was added to each and shaken with the silica. The latter was brought down by centrifugation. A silica blank for PCA was obtained by scraping an amount of silica from a blank part of the plate equal in weight to that containing presumed PCA and treating it similarly. Silica blanks for presumed VA and presumed IVA were similarly prepared.

The spectrum of each test solution was obtained in an Optica Double beam spectrophotometer, which compared the spectra in two cells, one a test cell, the other a reference cell. In each case

the reference cell contained the blank corresponding to the solution in the test cell. This manoeuvre ensured that substances in the silica (or the solvents) did not interfere with results. The spectra thus obtained were compared with the following:-

1. those obtained for 0.0001 M methanolic solutions of authentic PCA, VA and IVA.

2. the data given by Nakagawa et al. (1964) for authentic PCA, VA and IVA.

Nakagawa et al. (1964) described a shift in peak maxima following the addition of a drop of 1% methanolic aluminium chloride to the solutions being examined. Use of this method allowed new comparisons to be made between spectra obtained from presumed metabolites and

3. those obtained by similar treatment of 0.0001 M methanolic solutions of the authentic compounds.

4. the data given by Nakagawa et al. (1964).

In all cases close agreement was found for peak maxima.

The identification of vanillylglycine. The identification of metabolite 4, vanillylglycine presented more difficulty as the authentic compound was not available commercially. Vanillylglycine appeared on paper chromatograms derived from unhydrolysed urine as a third spot (see Fig. 26B) which, using

the chloroform : glacial acetic acid : water solvent system (1(a) Table XIII) had an Rf on paper chromatograms of 0.14. This agrees satisfactorily with the value of 0.10 found for this compound by Booth et al. (1957).

The third spot was identified as vanillylglycine by the methods for identifying isovanillylglycine used by Price (1969).

Three important points need to be made.

1. That complete acid hydrolysis of vanillylglycine (carried out on a solution containing 15 ml. of urine and 3 ml. concentrated hydrochloric acid) required 15 lb./sq. in. pressure for 1½ hours; thus if the above solution was simply boiled on a water bath for 30 minutes the third spot continued to appear on subsequent chromatograms indicating incomplete hydrolysis. It may be mentioned that Hill et al. (1959) noted that vanillylglycine was "rather stable to acid hydrolysis."

2. That the third spot almost certainly contained small amounts of isovanillylglycine also. The author has shown in a study on the metabolism of isovanillic acid (Price, 1969) and in the present study that in the chromatographic conditions specified above, vanillylglycine and isovanillylglycine behaved identically with respect to Rf and staining with sulphanilic acid (both yielding reddish-pink spots). For both substances the given conditions ensured complete acid hydrolysis.

3. Vanillylglycine was estimated quantitatively by subtraction of free from total VA (see p 198 and 205).

The conjugation of PCA (other than by O-methylation). Dodgson and Williams (1949) in a study in rabbits, found an increase in glucuronic acid and (to a lesser extent) in ethereal sulphate excretion after PCA had been administered. This led them to conclude that glucuronide and sulphate conjugation of PCA takes place in the rabbit.

Clarke et al., (1956) found an increase in urinary glucuronide excretion of about 0.5 G/day after they had administered 5.33 - 6 G. of PCA per day to three subjects with rheumatic fever (the sulphate excretion did not rise suggesting that sulphate conjugation was not occurring). If this increase were due entirely to PCA glucuronide formation it would correspond to the conjugation of approximately 0.4 G. of PCA ($0.5 \times \frac{\text{molecular weight PCA}}{\text{" " glucuronic acid}}$), or 1/15th of the ingested dose. (Since the amount of conjugation may be to a considerable extent dose-dependent, this figure of 1/15th cannot be transposed directly to the present study.) It would seem therefore, that PCA glucuronide, at this dosage of PCA can be regarded as no more than a minor metabolite of PCA.

In the present study, two observations were made (neither by any means conclusive) which would make it likely that the same applies when a lower dosage of PCA is used:-

1. PCA spots were obtained both from hydrolysed and non-hydrolysed urines, (the latter during the measurement of

unconjugated VA, see Fig. 26B). On a number of occasions measurements were made of spot size when 10 µL spots deriving from the same volumes of the same test urine were compared, one spot deriving from hydrolysed, the other from unhydrolysed urine. One would have expected that PCA glucuronide should yield to acid hydrolysis and that the PCA spot would, therefore, be larger on the hydrolysed specimen. This was not noticeable in the studies made.

2. PCA glucuronide (having a free OH group) might be expected to stain with sulphanilic acid. No extra unexplained spot was found on chromatographs obtained from unhydrolysed urine, nor was an extra peak found on the gas chromatograms derived from unhydrolysed urine, although it is possible that the extraction conditions might not extract PCA glucuronide or might extract it very incompletely.

To summarise, the possibility exists that PCA forms a glucuronide in man, but this metabolite seems unlikely to be more than a minor one.

This metabolite has not been further considered in the present work, and the term PCA is used, without qualification, to indicate the amount of PCA which was found in urine extracts which were developed from hydrolysed urine. It is worth adding that the studies on methacetin suggest that there is no important

difference in glucuronide formation between normal and schizophrenic subjects (it will be recalled that paracetamol glucuronide is quantitatively much the largest methacetin metabolite in man).

The 4-dehydroxylation of PCA in man. Tompsett (1959) suggested 3-hydroxybenzoic acid (formed by 4-dehydroxylation) as an important metabolite of PCA in man. None of the techniques used in the present study support the occurrence of this metabolite; in particular the gas chromatographic studies would appear to rule it out. The evidence for this statement will be more easily understood in the context of the gas chromatography section and it has been included in that section (p. 235).

Methods. 2 Quantitative measurements.

2(a) Estimation of PCA and VA in urine.

1. The procedure was carried out in duplicate throughout.

2. An amount of urine varying between 3 ml. and 15 ml., (the former with very small three hour samples (< 100 ml.) and the latter with samples of over 350 ml.) was placed in a universal container. Where less than 15 ml. urine was used the volume was made up to 15 ml. with distilled water. Standard solutions (carried in duplicate routinely through every batch of estimations) contained 10 mgm. PCA and 5 mgm. VA in 15 ml. water.

3. 3 ml. concentrated Analar hydrochloric acid were added and the universal container was screwed up tightly and subjected to

autoclaving for 1.5 hrs. at a pressure of 15 lbs/sq. in.

4. The entire 18 ml. was transferred to a 50 ml. quickfit flask (stoppered with a ground glass stopper). The universal container was then rinsed with small amounts of distilled water to ensure complete transfer.

The pH was adjusted to lie within the range 1.2 to 1.8; this was done in two stages - 7.6 ml. of 4 N NaOH was first added and then this alkali or 3 N hydrochloric acid was added dropwise until the pH lay within the required range, as determined by the use of a pH meter. 10 G. Analar sodium chloride (enough to saturate the solution) and 25 ml. Analar ethyl acetate were added and the flask was stoppered and shaken mechanically for 20 minutes.

5. The ethyl acetate was decanted into a boiling tube and 15 ml. pipetted into a flask containing 3 ml. 10% aqueous $NaHCO_3$. This flask was shaken for five minutes.

6. 2 ml. of the lower (inorganic) phase were then transferred into a 10 ml. Exelo tube and 0.5 ml. 5 N HCl added (cautiously because of effervescence). The tube was shaken until effervescence entirely ceased. 0.5 ml. Analar ethyl acetate was added and the tube stoppered with a ground glass stopper and shaken by hand for a total time of about two minutes.

7. As much as could be removed of the ethyl acetate was taken off with a Pasteur pipette into a small tube (which could be

fitted tightly with a plastic stopper). This tube contained the "Ethyl acetate extract" used for VA, IVA, PCA and VRA estimations; for VA and PCA estimations the extract was used immediately (v.i.), since ethyl acetate is volatile and readily evaporates even from a stoppered tube.

8. One-way ascending paper chromatography was carried out in the following manner.

Solvent: The lower (organic) phase of a mixture of Analar chloroform 2 parts, Analar glacial acetic acid 1 part and distilled water 1 part, which had been mixed vigourously for several minutes in a separating funnel and then allowed to separate over night, was the solvent used.

This solvent, which was used once only, was placed in a plastic tray within a glass chromatography tank, the lid of which was greased with silicone grease and weighted down. This was done about two hours before the tank was required for use.

Paper: 10" x 10" Whatman No.1 squares were used.

9. A line parallel to one side and 2 cm. from it was ruled and 10 μL of the ethyl acetate urine extracts were placed at three points on this line, spaced at 4.5 cm. intervals. Two applications including the middle one were derived from one specimen of urine; the third was from the duplicate specimen. Application to the paper was made by means of a 10 μL Kirk Type E-mil pipette.

10. Ascending chromatography was carried out for two hours, the solvent front ascending to a level of 14 - 17 cm. above the starting line. The paper was dried at room temperature, without a fan, in a fume cupboard.

11. The precise positions of the solvent front and of the separated constituents of the ethyl acetate extracts were then obtained by examining the paper in a dark room under a 254 mμ. U.V. light source. Both PCA and VA produced dark spots on the background flourescence of the paper under these conditions. In addition, the track of ascent of the extracts of urine was marked by faint fluorescence; this was not the case when aqueous standards were used.

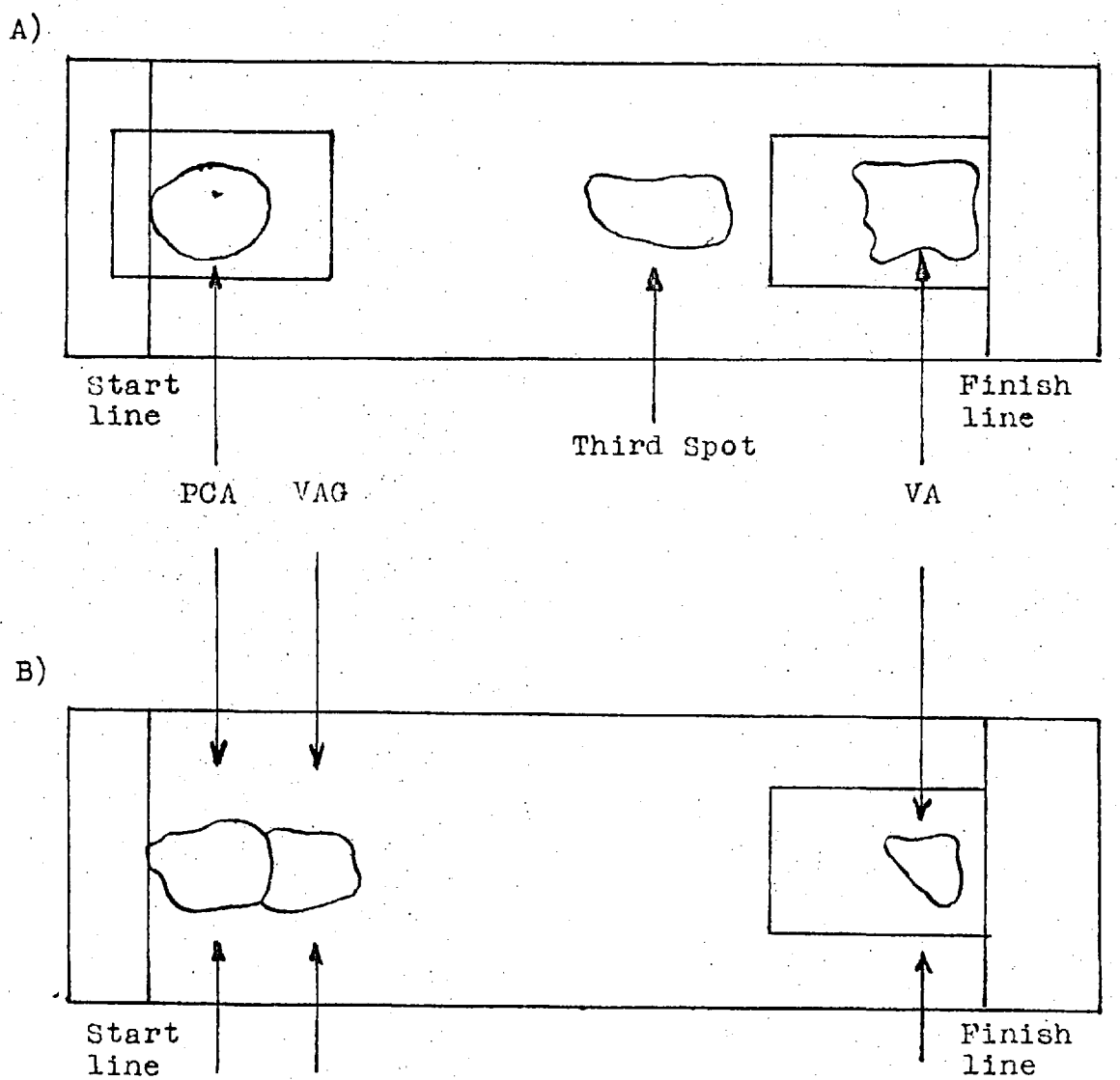
12. The track corresponding to the ascent of the middle of the three spots which had been applied was cut out in its entirety and stained with sulphanilic acid reagent under the conditions given by Smith (1960).

This enabled the position of the PCA spot and the VA spot to be localised, the former being of a light brown, the latter of a brilliant orange colour. Evidence of identity of these substances is presented in a previous section.

Rfs obtained (mean of 10 determinations using different urines)

Protocatechuic acid	:	0.11
Vanillic acid	:	0.92

Figure 26. Drawing of paper chromatograms for PCA & VA estimation: PCA & total VA (free & conjugated) were measured from the upper and free VA from the lower Chromatogram.



Colours Fawn Red-pink Orange

Solvent System. Lower (organic phase) of Chloroform: Glacial Acetic Acid: Water :: 2 : 1 : 1

Staining. Sulphanilic Acid Stain. (from Smith, 1960.).

Finish line = Solvent front.

On most chromatograms a third spot of a brownish yellow colour was present on the paper, having an Rf value of 0.64. This was present as the only sizeable spot on all six chromatograms derived from the six pretest urines obtained from normal subjects. It was clearly not a PCA metabolite. The clear chromatographic separation of this spot from the spots of PCA and VA meant that its presence did not interfere with the elution described below.

13. The two unstained strips from each paper were cut for elution in the following manner. Two areas of paper were removed of size 5 cm. x 4 cm., with the 5 cm. in the long axis of the strip, in each and every case. These were always large enough to include the total area corresponding to the PCA and VA on the sulphanilic acid-stained paper (see Fig 26 A). A piece of chromatography paper through which the solvent had ascended but through which no urinary extracts had run, served as a blank. This was three times as large as the 5 x 4 cm. eluate rectangles and three times as much water was subsequently added to it so as to provide enough solution for filling the three cuvettes used for the spectrophotometric estimation described below.

14. The oblong pieces of chromatography paper were cut into strips so that they could be placed in 15 ml. stoppered exelo tubes.

15. 5 ml. of distilled water was added to the tubes containing

the test strips (and 15 ml. to the tube containing the paper blank).

The tubes were stoppered, shaken vigorously for one minute, and the contents filtered through Whatman No.1 paper into a second set of tubes.

16. The colour used for spectrophotometric estimation was developed by means of the Emerson reaction (Emerson et al., 1943). The precise conditions were derived from those of Sumere et al. (1961) who used the following combination of reagents:-

0.3 ml. 0.1 N NaOH

0.2 ml. 1% 4-aminoantipyrine

0.1 ml. 5% K_3 $Fe(CN)_6$

to which they added aqueous solution of phenolic acids, to a total of 10 ml. This method was adapted for the present work as follows:-

a) 3 ml. of the solution obtained from the filtration of the paper eluates was used, so that a total volume of 3.6 ml. resulted, a convenient amount for use with standard 4 ml. cuvettes.

b) The Emerson reaction is highly sensitive to pH (Ettinger et al. 1951). The chromatograms having been developed in a solvent system which included glacial acetic acid gave an acid solution when eluted with water. Subsequently 0.3 ml. 0.1 N NaOH proved inadequate as an alkalinizing agent.

Colour produced by standard amounts of PCA and VA was developed at a variety of pH values. For PCA highest values were produced at pH 10.1 to 10.8, and for VA at pH 9.4 to 10.2. Over these ranges, in each case, very little change in values was noted.

The buffers used were:-

1. For Vanillic acid (pH 10.0)

   61.5 ml M $KHCO_3$

   31.75 ml M NaOH

   Water to 100 ml.

2. For Protocatechuic acid (pH 10.6)

   45.3 ml M $KHCO_3$

   35.3 ml M NaOH

   Water to 100 ml.

Elutions from the paper (3 ml.) when added to 0.3 ml. of these buffers (and to the other reagents) were shown repeatedly to have a final pH within the pH ranges quoted above as yielding maximum colour.

c) As the final colour produced on adding the potassium ferricyanide reagent fades with time, the following procedure was adopted.

3 ml. of eluate were placed in an Exelo tube and 6.3 ml. of the appropriate buffer and 0.2 ml. of 1% 4-aminoantipyrine

solution were added. Just prior to placing the solutions into cuvettes for reading, 0.1 ml. of 5% $K_3Fe(CN)_6$ was added. The tubes were stoppered, shaken and transferred immediately to cuvettes for reading. Maximal colour was shown by experiment to be produced immediately on mixing, fading thereafter.

17. Spectrophotometric estimates were carried out on a Unicam 500 spectrophotometer. The colour obtained with standard solutions of VA gave an absorption peak at 490 mμ ; the colour obtained with PCA gave an absorption peak at 510 mμ.

It was decided, as a matter of convenience, to carry out all readings at 500 mμ. The 'blank' against which cuvettes were zeroed was the yellow solution derived by the addition of the reagents using either buffer to eluates of paper (as described above). Both buffers gave the same blank setting.

2(b) <u>The procedure for estimating free Vanillic Acid</u>.

1. Duplicate aliquots of 15 ml. of urine were taken and 10.6 ml. of water added; this corresponds to the 3 ml. of concentrated HCl and the 7.6 ml. of 4N NaOH added when acid hydrolysis was carried out. (Stages 4 - 7 were as above).

2. Because so little of the Vanillic acid is unconjugated, chromatographing 10 μL of ethyl acetate extract was found to yield very low spectrophotometric readings. Therefore 30 μL instead of 10 μL was spotted. Only the VA spot was eluted, the

chromatographic strip having the appearance shown in Fig. 26 B, (lower drawing). The procedure was otherwise identical with that described above.

2(c) The parallel lines experiment. This experiment is similar to the one employed in paracetamol estimation, and has the purpose of showing that the presence of constituents of normal urine does not interfere with the chemical method of estimation.

Materials:

Four lines were constructed for VA and four for PCA, the urine used having been collected from a subject who had received an oral dose of 1,000 mgms. of PCA and who had thereafter collected urine for three hours , the conditions of the PCA test (p. 237) being adhered to throughout.

Methods:

The standard solution contained 100 mgms. of PCA and 100 mgms. of VA per 100 ml. distilled water.

Universal containers were made up as follows, all volumes being in ml.

Bottle No.	1	2	3	4	5	6	7	8
Urine	0	0	0	0	0	0	0	0
Std.PCA+ VA	2.5	2.5	5	5	7.5	7.5	10	10
Water	12.5	12.5	10	10	7.5	7.5	5	5
Conc. HCl.	3	3	3	3	3	3	3	3
Total	18	18	18	18	18	18	18	18

Bottle No.	9	10	11	12	13	14	15	16
Urine	5	5	5	5	5	5	5	5
Std.PCA+ VA	2.5	2.5	5	5	7.5	7.5	10	10
Water	7.5	7.5	5	5	2.5	2.5	0	0
Conc. HCl.	3	3	3	3	3	3	3	3
Total	18	18	18	18	18	18	18	18

In the second part of the experiment various amounts of urine were set up so as to construct a third line for both VA and PCA. Finally a fourth line was obtained by adding a fixed quantity of the same PCA+VA standard.

Bottle No.	17	18	19	20	21	22	23	24
Urine	2.5	2.5	5	5	7.5	7.5	10	10
Std.PCA+ VA	0	0	0	0	0	0	0	0
Water	12.5	12.5	10	10	7.5	7.5	5	5
Conc. HCl.	3	3	3	3	3	3	3	3
Total	18	18	18	18	18	18	18	18

Bottle No.	25	26	27	28	29	30	31	32
Urine	2.5	2.5	5	5	7.5	7.5	10	10
Std.PCA+ VA	5	5	5	5	5	5	5	5
Water	7.5	7.5	5	5	2.5	2.5	0	0
Conc. HCl.	3	3	3	3	3	3	3	3
Total	18	18	18	18	18	18	18	18

All bottles were stoppered, autoclaved and carried through the estimation procedure described above (section 2(a)).

Results:

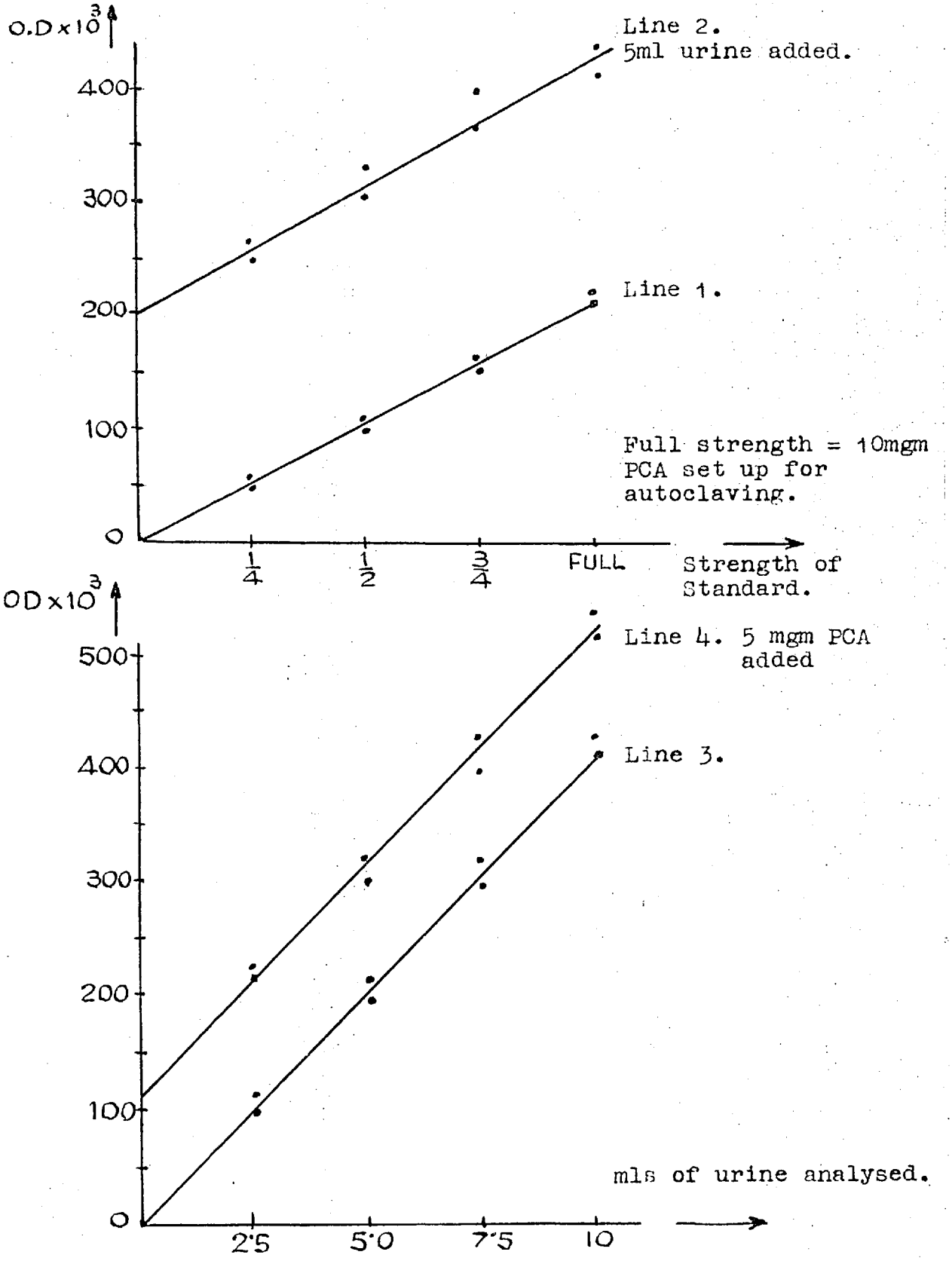
Spectrophotometric readings were obtained as follows. For convenience, all values have been multiplied by 10^3.

Bottle No.	Line 1 PCA	Line A VA	Bottle No.	Line 2 PCA	Line B VA
1	52	208	9	266	662
2	58	192	10	248	711
3	99	399	11	306	891
4	109	368	12	333	828
5	160	652	13	367	1076
6	151	580	14	400	1035
7	223	769	15	440	1222
8	208	820	16	411	1312

Bottle No.	Line 3 PCA	Line C VA	Bottle No.	Line 4 PCA	Line D VA
17	101	239	25	227	635
18	115	258	26	222	644
19	198	486	27	302	870
20	214	498	28	322	897
21	300	778	29	399	1128
22	321	738	30	432	1178
23	406	979	31	539	1390
24	433	1031	32	520	1324

Figure 27a.

Parallel Lines Experiment
for Protocatechuic Acid (P.C.A.)



Statistical Evaluation:

Using the data given above, the following calculations were obtained for lines 1 - 4, 'b' being the slopes of the lines, and 'a' the y axis intercepts; Fig.27a illustrates the results obtained.

	Slope of line=b	inter-cept= a	Se (b)	$\frac{b}{Se(b)}$	p for 6 d.f.	Se (a)	$\frac{a}{Se(a)}$	p for 6 d.f.
Line 1	213.2	-0.75	8.72	24.4	<0.001	5.97	0.126	N.S.
Line 2	227.8	204.0	21.64	10.5	<0.001	14.82	13.8	<0.001
Line 3	41.56	1.25	1.52	27.27	<0.001	13.20	0.09	N.S.
Line 4	40.74	116	1.86	21.9	<0.001	12.7	9.1	<0.001

There was no significant difference in the slopes of lines 1 and 2 ($t = 0.62$, $p>0.1$).

Line 1 did not have a significant intercept on the y axis.

Line 2 had a significant intercept on the y axis (of 204.0).

There was no significant difference between the slopes of lines 3 and 4 ($t = 0.34$, $p>0.1$).

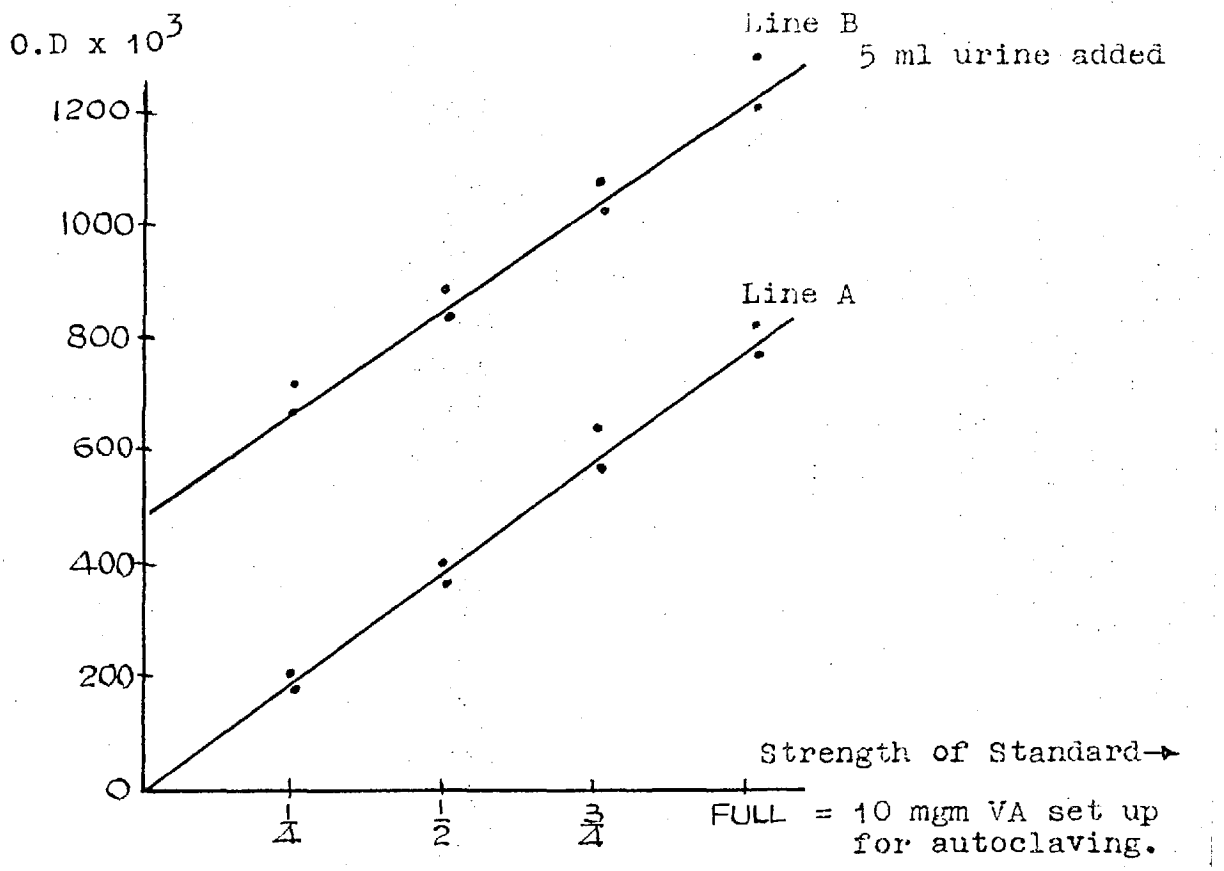
Line 3 did not have a significant intercept on the y axis.

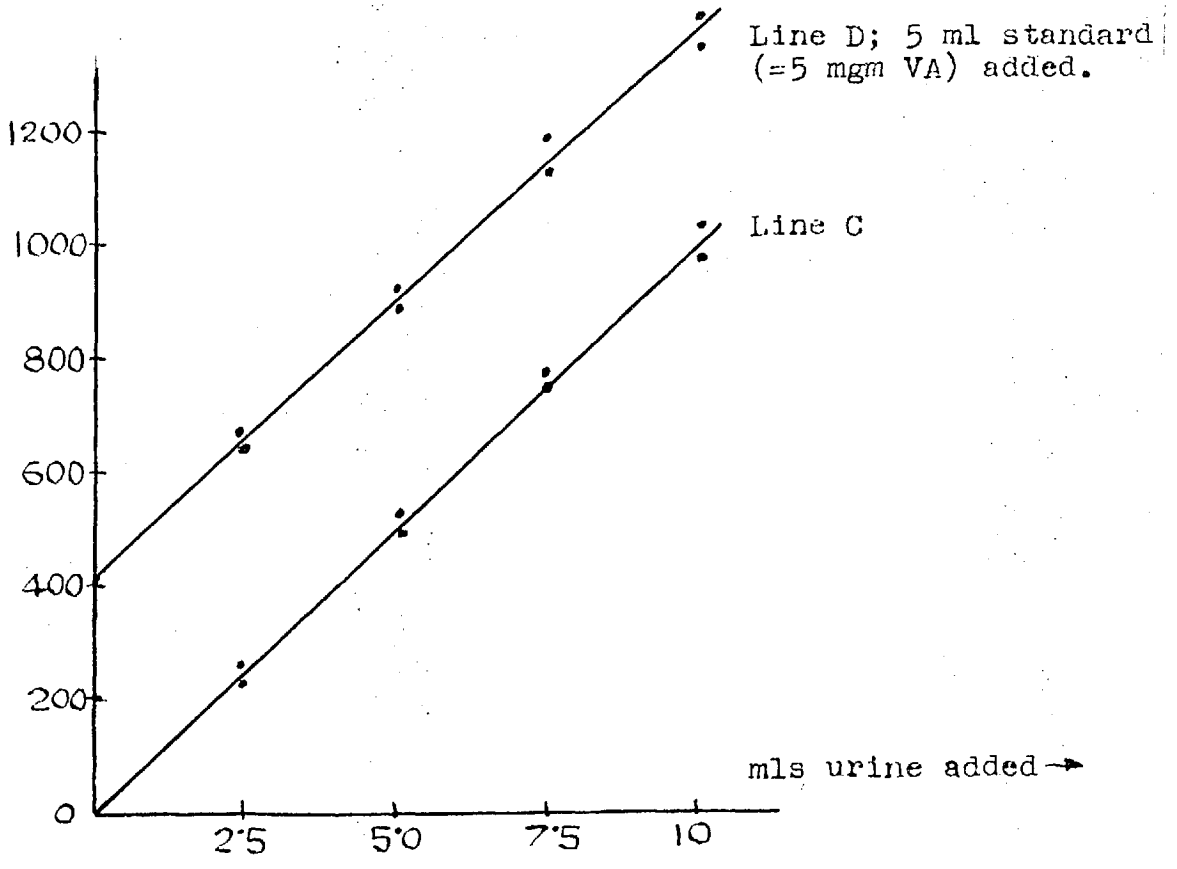
Line 4 had a significant intercept on the y axis (of 116).

This corresponded to the addition of 5 ml. of standard; on this basis 10 ml. of standard would give an intercept of 232; this figure agreed fairly well with the figures obtained for the slope of lines 1 and 2, i.e. 213.2 and 227.8.

Figure 27b.

Parallel Lines Experiment for Vanillic Acid. (VA).





Furthermore, the intercept of line 2, which represented the effect of the addition of 5 ml. urine is 204.0; on this basis 10 ml. of urine would give an intercept of 408.0 or 40.8/ml. This letter figure agreed well with the slope of line 3 (41.56) and of line 4 (40.74).

Similarly, for lines A - D (all for Vanillic acid :Fig. 27b)

	Slope of line =(b)	Inter-cept =a	Se (b)	$\frac{b}{Se(b)}$	p for 12 d.f.	Se (a)	$\frac{a}{Se(a)}$	p for 12 d.f.
Line A	801	-5.5	29.8	26.9	<0.001	20.45	0.269	N.S.
Line B	775	483	48.5	16.0	<0.001	33.2	14.5	<0.001
Line C	101.4	-8	2.924	34.67	<0.001	17.97	0.445	N.S.
Line D	96.9	403	3.816	25.39	<0.001	26.13	15.4	<0.001

Student's t for the comparison of slopes for lines A and B = 0.446
Thus the difference between the slopes of these lines was not significant. Line B had a significant intercept on the y axis :- 483.
For the comparison of slopes for lines C and D, t = 1.02.
There was no significant difference between the slopes of lines C and D.
Lines A and C did not have a significant intercept on the y axis.
Line D had a significant intercept on the y axis :- 403.
This intercept corresponded to the addition of 5 ml. of standard

to all the bottles from which line D is derived. 10 ml. of standard would thus correspond to an O.D. of 806. This agreed well with the slope calculated for line A (801) and line B (775), which represented this amount of standard.

Furthermore the intercept of line B is 483 and this represented the effect of adding 5 ml. of urine; on this basis 1 ml. of urine would yield an O.D. of 96.6. This figure agreed well with the slope of line C (101.4) and of line D (96.9).

Conclusion.

Evidence of interference by substances in the urine with the chemical estimation of PCA and VA was not detected in this experiment.

2(d) Interference by substances in urine other than protocatechuic acid metabolites. Human urine contains a large number of phenolic acid compounds (Armstrong et al., 1956 and Williams and Sweeley, 1964).

That a good many of these are of dietary origin is suggested by the work of Studnitz et al., (1964) who demonstrated that the number of urinary phenolic acids falls sharply when subjects are placed on to a glucose diet. These authors considered that ten phenolic acids were derived from endogenous sources.

For the present study it was impracticable to maintain acutely disturbed schizophrenics on a glucose free diet for several days before administering protocatechuic acid.

Food stuffs known to be important sources of phenolic acids were excluded from the diet on the day before the test. A list of excluded foodstuffs was prepared from a paper on plant phenols in food (Bate-Smith, 1959), and comprised the following:-

All fruit (All drugs)

Spinach, carrots,

Beer, Coca Cola, coffee, fruit drinks, cocoa,

Custard, Blancmange, ice cream.

Tea and milk were permitted drinks.

In drawing up this list, particular attention was paid to those foodstuffs containing or yielding ferulic acid and vanillic acid. For example, coffee was excluded because vanillic acid and ferulic acid are metabolites of caffeic acid, a constituent of coffee (Booth et al., 1957). VA and ferulic acid run together in the chloroform/glacial acetic acid/water solvent system used for chromatography. Furthermore, ferulic acid reacts with the Emerson reagent, as one would expect from its phenolic structure.

It must be emphasised that the dietary restrictions imposed in the test were intended to reduce rather than abolish interference from dietary phenolic acids, the quantities of which could be anticipated to be relatively small. Thus Williams and Sweeley (1964) give a list of the amounts of various phenolic acids produced per day on a normal diet. Eight acids are listed

as being excreted in quantities as high as 20 mgm/day and seven others in amounts of up to 5 mgm/day. In a three hour collection the larger figure will reduce proportionately to 1/8th, i.e. 2.5 mgm for each of the eight acids. The amounts of PCA and vanillic acid excreted in the test sample are of the order of 80 - 250 times this figure of 2.5 mgm.

Furthermore, not all the chromatogram was used for elution. A sizeable area which will contain a number of phenolic acids was discarded.

Materials and Methods.

The only safe way to evaluate interference was by measuring it. In order to do this, six of the volunteer subjects (chosen at random) were asked to collect the urine that they passed just prior to the administration of protocatechuic acid. These subjects were not forewarned in any way and therefore did not have the opportunity to adhere to the dietary restrictions more closely than they might otherwise have done. Pretest specimens were collected from all but one of the schizophrenics tested. They were examined for vanillic and protocatechuic acids using the usual methods, except that 30 μL was spotted on to the paper instead of 10 μL, in order to produce larger spectrophotometric readings.

From these pretest urines the amount of interference was

calculated on the basis of how much VA and PCA would have been calculated as being present in pretest urine containing an identical amount of creatinine to that found in the test urine (see Table XIV). Since the dietary restrictions were in force until the end of the three hour PCA test period, there is no reason to suppose that these results should be invalidated by increased contamination with phenolic acids of dietary origin excreted during this period; the period of continued fasting should act in the opposite direction - i.e. towards decreased contamination although this effect will probably only be slight.

Results:

TABLE XIV . Interference by urinary phenolics, calculated from pretest urine specimens.

Normal Subject No.	Excreted in test PCA mgm.	*Pretest urine content of PCA mgm.	**Excreted in test VA mgm.	*Pretest urine content of VA mgm.
13	393	1.7	353	2.5
14	315	1.2	289	1.4
19	451	2.8	299	2.3
20	580	1.4	310	1.5
39	498	1.6	352	1.5
42	595	1.5	322	1.6

Schizophrenic Subject No.	Excreted in test PCA mgm.	*Pretest urine content of PCA mgm.	**Excreted in test VA mgm.	*Pretest urine content of VA mgm.
31	551	2.3	277	1.5
32	274	2.1	209	0.9
33	457	2.6	214	1.2
34	170	0.4	149	1.4
35	493	3.2	209	2.8
36	298	6.2	160	2.5
37	238	2.8	199	1.5
38	515	4.7	210	2.5
39	577	5.8	244	4.5
40	437	1.2	294	2.2
41	471	0.5	184	3.6
42	267	6.0	205	6.2
43	375	4.2	172	3.0
44	423	4.9	225	4.2
45	696	11.8	377	8.5
46	585	3.3	410	1.1
47	No specimen : totally uncooperative before the test started.			
48	404	7.8	157	3.2
49	491	6.3	347	5.6
50	262	2.9	169	0.7
51	548	13.9	331	3.9

*Based on the amount calculated as that which would have been present in a sample containing an equal amount of creatinine to that in the 3 hour test sample.

** VA is expressed here and elsewhere as the amount of PCA from which it derives. This applies (elsewhere) to IVA also.

** These values for VA are not corrected for the presence of IVA.

All results depicted in the Table have been calculated by the same method, i.e. from paper chromatography results only without reference to the $\frac{VA}{IVA}$ ratio.

Statistical Evaluation. a) Normal Subjects.

The greatest amount of interference in any measurement is 0.77%, and the average amount for PCA estimations is 0.37% and for VA estimations 0.56%.

Statistical Evaluation. b) Schizophrenic Subjects.

The greatest amount of interference is 3% in any one measurement - the average for PCA is 1.1% and for VA is 1.3%.

Conclusion.

The amount of interference in VA and PCA measurement is of low order. It has been ignored in subsequent calculations.

2(e) The method used for the estimation of the ratio VA/IVA in standards and urine extracts. The method used involved the use of thin layer chromatography (T.L.C.) to separate VA from IVA. The chromatograms were stained with the Folin-Ciocalteu reagent and the amount of colour was quantified by using a scanning apparatus.

Materials.

1. Precoated T.L.C. plates were obtained from Merck Limited.

These were 20 cm. square with a 0.25 mm. Silica Gel layer impregnated with a fluorophor which, when activated by 254 mμ light, produced a green fluorescence. These plates gave best separation of VA from IVA without further activation.

2. Benzene : acetic acid : water :: 2 : 2 : 1 (upper phase) proved a satisfactory solvent system. The reagents (Analar grade) were shaken in a separating funnel, left overnight and the lower phase discarded. The solvent thus prepared can be made up in bulk; it behaves well after storage at room temperature for a period of several weeks.

Method of Chromatography.

Applications were made along a starting line ruled 2 cm. up from the edge of the plate.

It will be recalled that duplicate ethyl acetate extracts were made from each test urine. For the present estimations these required dilution approximately 3 - 6 fold in order to yield spots of suitable size. Preliminary thin-layer chromatography indicated the amount of dilution required. Two applications, one from each duplicate diluted solution were made; one of 2 μL, the other of 1 - 1.5 μL (see Fig. 28). This was done in order to obtain values for the VA/IVA ratio derived from different absolute quantities of VA and IVA.

Nine spots were applied to each plate : 5 standards and two

samples from each of two different test subjects. All applications were made with 2 μL micropipettes.

Standards were made up as follows, the figures representing μG/2 μL of ethyl acetate (Table XV).

TABLE XV . The amounts of VA and IVA used as standards, (μG/2 μL ethyl acetate).

Standard No.	VA	IVA
1	2.0	0.3
2	1.5	0.225
3	1.0	0.15
4	0.667	0.1
5	0.5	0.075

Figure 28 showing the arrangement of spots on the thin-layer plate.

4	A	2	B	3	A	5	B	1
2μL	2μL	2μL	1.3μL (approx)	2μL	1.3μL (approx)	2μL	2μL	2μL

1 - 5 = Standards as in Table XV above

A, B = diluted ethyl acetate extracts derived from urine from subjects A and B.

The tank used measured 25 cm x 25 cm x 5 cm. Preliminary equilibration of the tanks with solvent was found to be disadvantageous

(leading to streaking and poor separation) and was not used.

The development time of 40 minutes gave a solvent ascent of about 15 cms.

After development the plates were removed from the tank and dried in an oven at 120°C in the horizontal position for five minutes, followed by five minutes drying in the vertical position, the latter facilitating the evaporation of the last trace of solvent.

Staining:

After cooling to room temperature, plates were sprayed with 10% Na_2CO_3. Spray bottles capable of delivering very fine sprays were used (throughout). Spraying continued until the plates were just saturated. Plates were then dried for ten minutes at 120°C; cooled and sprayed with a freshly made up mixture of one part of Folin and Ciocalteu's phenol reagent (obtained as a stock solution from British Drug Houses) and two parts of water, again until the plates were just saturated.

The plate was then stood for ten minutes horizontally on the bench and for 10 minutes horizontally in the oven at 120°C.

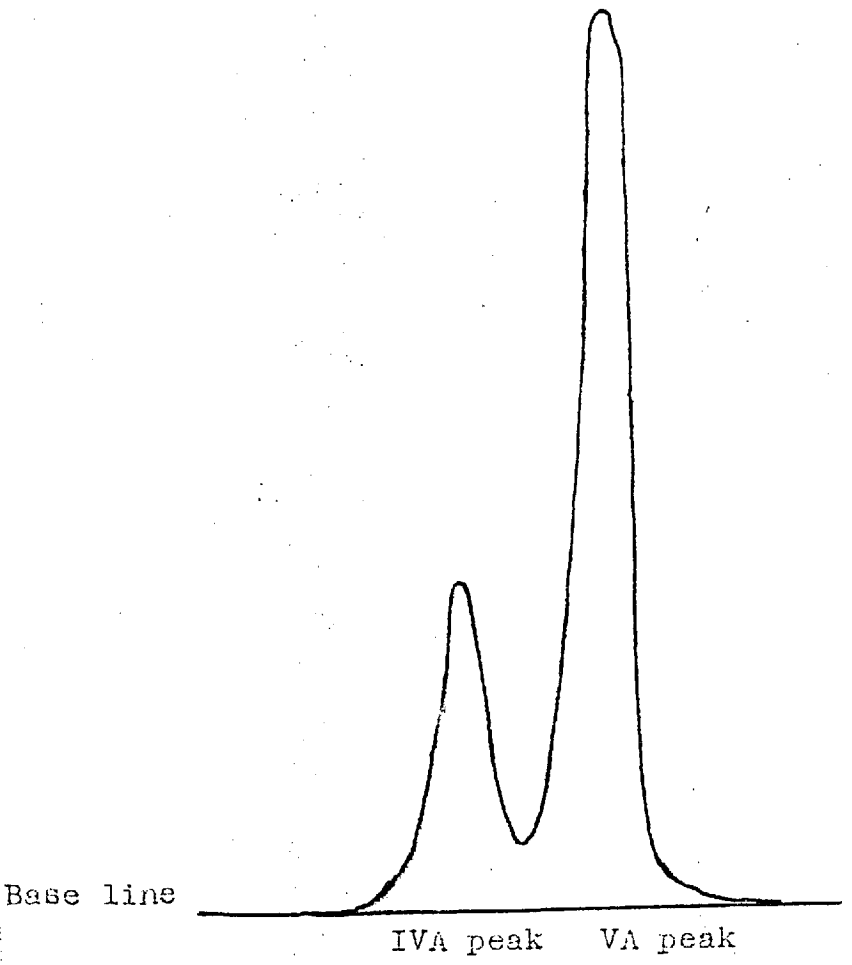
Colour Measurement:

The blue spots thus obtained were quantified in a Chromoscan Mark II (Joyce, Loebl & Co., Ltd.) fitted with a thin layer attachment. Filters of 595 mμ were inserted and slit sizes of

Figure 29.

The Appearance of a Chromoscan Tracing.

This figure is a full scale tracing from one of the records obtained on a TLC plate deriving from a urine extract.



9 x 0.5 mm. in the Chromoscan and 20 x 1.5 mm. in the T.L.C. attachment were found suitable.

This apparatus produced a graphical record of the coloured spots on the plates. The slit width used in the T.L.C. attachment was slightly larger than the width of the VA spot on the highest standard (Standard 1). Appropriate dilution of VA spots from test specimens ensured that these VA spots were always smaller than that of Standard 1.

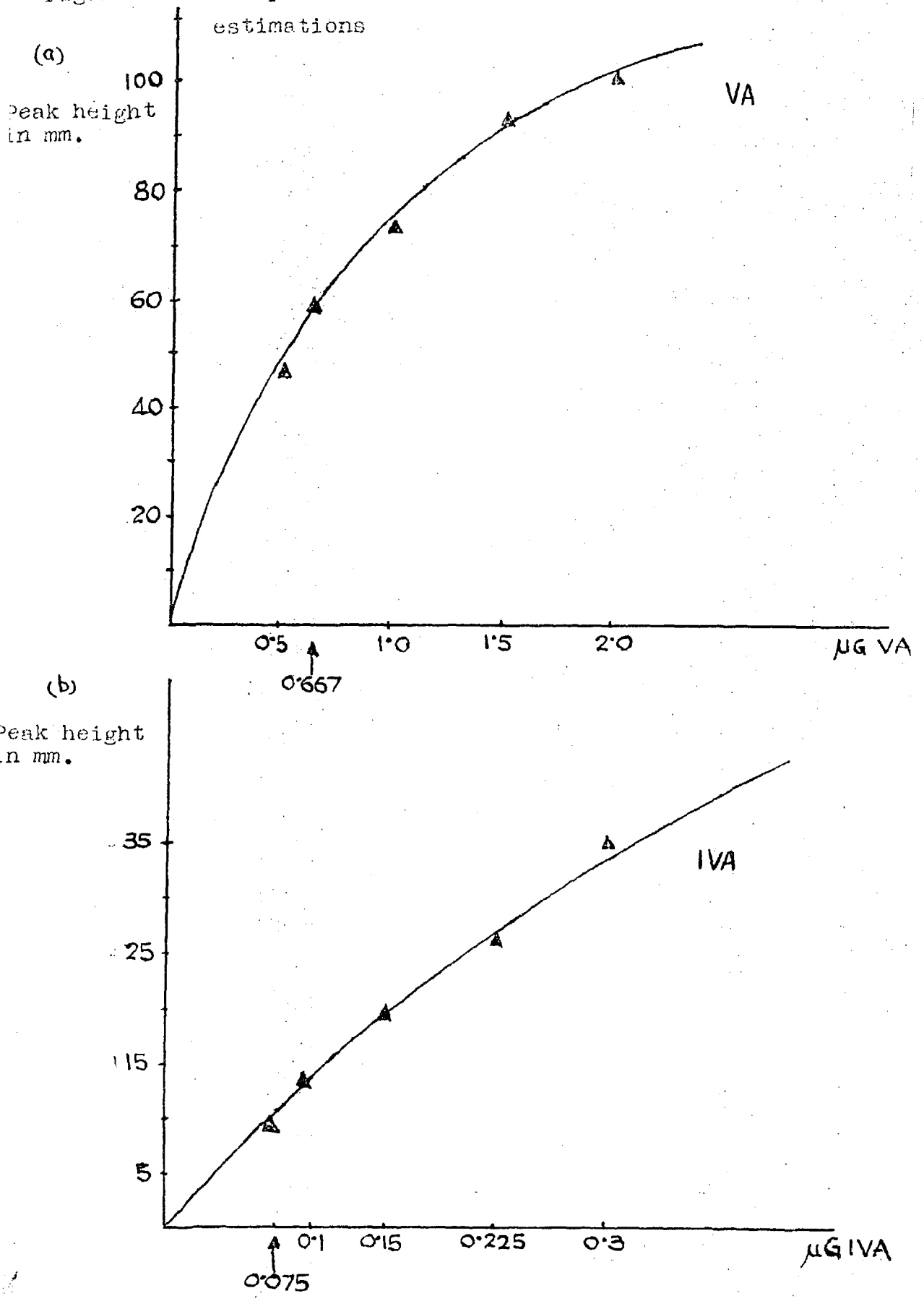
Three tracings were obtained of each of the nine chromatograms on each plate, the plate being recentred (that is, placed in position so that the middle of the slit passed over the middle of the spots) between each tracing.

On the tracings a flat base line with peaks corresponding to IVA and VA was obtained. The base line was drawn in under the peaks in line with the base line on either side of the peaks, as shown in Fig 29 . Perpendiculars were dropped from the summit of each peak to the base line, and peak height thus measured.

Method of Calculation of Results.

Two graphs were constructed, one for VA, the other for IVA:- On the ordinate the weight of IVA or VA in μG; on the abscissa, the peak height in millimetres. Using the conditions specified above the peak height for VA in Standard 1 (the most concentrated Standard) was approximately 110 mm., a height convenient to the

Figure 30 Examples of the standard curves used for VA & IVA estimations



range of stylus movement available on the Chromoscan.

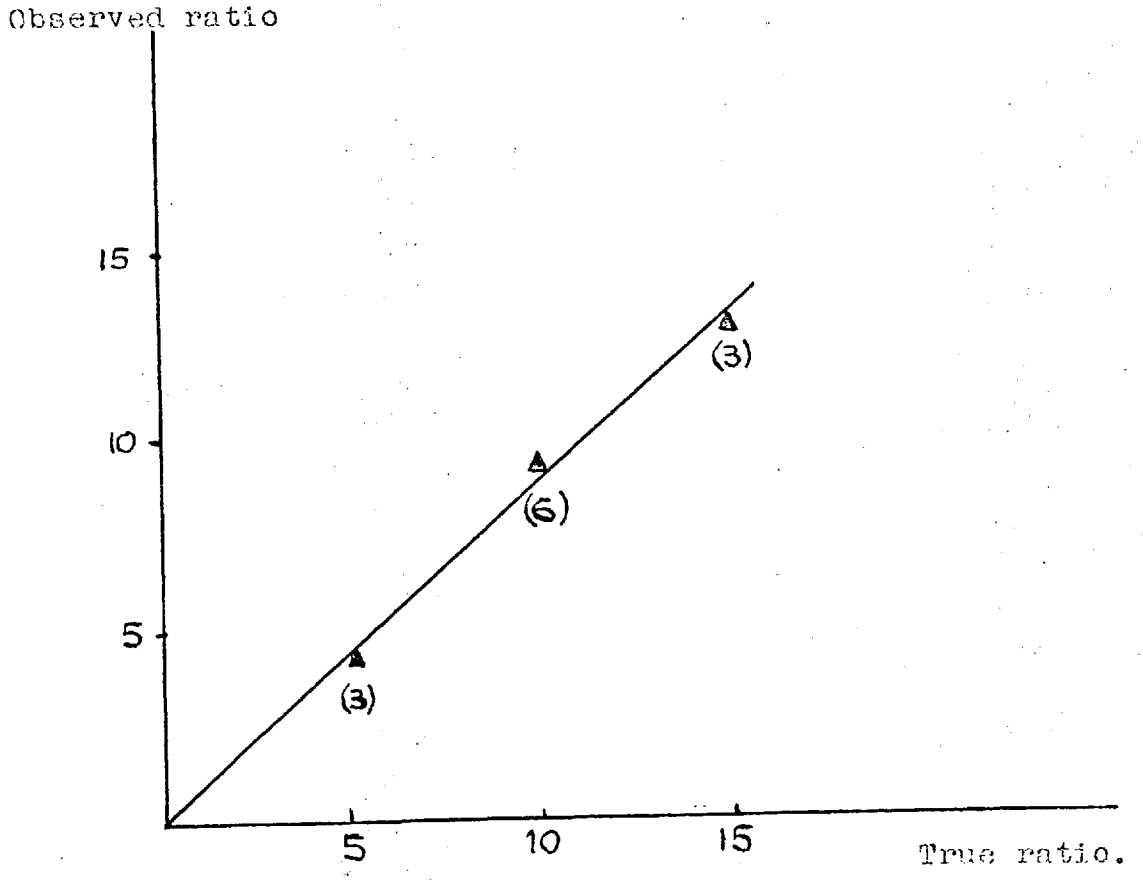
The graphs do not show a linear relationship between peak height and the amount of VA or IVA (see Fig. 30a and b). Linearity was not improved by plotting peak area against weight of material.

To derive the ratio for urine extracts, peak heights were measured and the amounts of VA and IVA read off from the graphs and the ratio thus obtained.

Ratios had to be corrected, because when 15 ml. standard solutions containing 10 mgm. of VA and 1 mgm. of IVA were taken through the entire extraction procedure (including acid hydrolysis), a ratio of 10 to 1 for VA to IVA was not obtained. Six such estimations gave ratios of 9.5, 8.7, 8.9, 9.3, 8.7 and 10.1 (mean 9.2) i.e. the method underestimated the true ratio. Part of the explanation for this is that the extraction of VA (92.5%)was slightly less than that of IVA (97.5%), with the methods employed. Different 15 ml. solutions of standards containing more VA (15 mgm. VA and 1 mgm. IVA) and less VA (5 mgm. VA and 1 mgm. IVA) were also analysed : the mean of three ratio estimations (13.8, 12.8 and 14.1) using the former was 13.3; the means of three ratio estimations (4.5, 4.1 and 4.5) using the latter, 4.4. All values given in the Tables of results have been corrected by dividing the original results by the slope of the line shown in Fig. 30c overleaf. This line has a slope of 10 units of y per 11.2 units of x. Its intercept on the y axis does not differ significantly from zero.

Figure 30(c)

The correction of the $\frac{VA}{TVA}$ ratio.



The subscribed numbers (in brackets) are the number of estimations from which each point has been derived, (by taking the mean of the estimations made).

Discussion.

The method given here had a number of disadvantages, the chief of which was the difficulty in obtaining satisfactory standard curves, particularly for VA. It was considered that the explanation for this difficulty usually lay in minor differences in staining; it was realised that the spray must be allowed to soak right through the thickness of the silica layer if the maximum colour is to be produced (i.e. all the VA and IVA must be allowed to react with the staining reagents). This amount of moisture carried the risk of diffusion of the acids (and, after staining, of the dye) away from the site at which they should be located, leading to a reduction in peak height.

A considerable wastage of plates resulted from these difficulties. Agreement between duplicate extracts was far from perfect and a difference between duplicates of 15% had to be accepted as a permissible upper limit : thus values for the ratio of 10 and 11.5 on duplicates were regarded as satisfactory; values of 10 and 11.6 were not accepted; in such a case two further values were obtained and subjected to the same procedure. It was not an uncommon experience to find that the mean derived from the second two values lay close to one of the first two values, indicating that the other value was the one in error.

An assessment of interference from non-PCA metabolites in the extracts.

The method was satisfactory in one important respect: that the area of the chromatogram to which IVA and VA normally migrated remained clear when pretest urine extracts were chromatographed (this applied to all the 26 available pretest urines). 10 μL of undiluted ethyl acetate extracts of these pretest urines was chromatographed and stained using identical techniques. Small peaks in the VA position measuring up to about 5 mm. were obtained (and might well have resulted from small amounts of urinary vanillic acid), but the IVA area of the chromoscan tracing remained free of measureable peaks. If one assumes that the contamination by phenolic acids not derived from administered PCA will not be much greater in test than in pretest urines (it will probably be somewhat less), then the contribution of these substances to 'test' VA peaks must be very small. This can be deduced from the following Table which refers to VA spots only:-

TABLE XVI Interference by urine phenolics, for VA in the VA/IVA ratio measurement.

	PRETEST	TEST
Dilution of ethyl acetate extract	Not diluted	3 - 6 fold
Size of application	10 μL	2 μL
Peak height	5 mm.	Average 80 mm.

3. Studies on Veratric Acid (3:4 dimethoxybenzoic acid : VRA) as a possible metabolite of PCA.

Note : The gas chromatography apparatus was only available for a limited time (at this time only schizophrenics 31 - 38 had been PCA tested). Results of the study being negative (VRA not being found), it has been decided to include them in this section rather than to incorporate them in the quantitative results presented in a subsequent section.

a. The extraction of VRA from aqueous solutions into ethyl acetate. If standard solutions of VRA are made up in water, acid hydrolysed and converted to ethyl acetate extracts by the usual methods and the residues evaporated under nitrogen and reconstituted with methanol, it was shown that recovery of veratric acid by these procedures was close to 100%. This procedure was conveniently carried out by examining the spectrum of the final methanolic solution in the Optica Double Beam Spectrophotometer.

b. The estimation of veratric acid in the presence of excess amounts of protocatechuic and vanillic acids. Veratric acid has no phenolic groups and therefore the estimation of this compound presented more of a problem than did that of VA and PCA, for it is on the reactivity of the phenolic groups that the methods of estimation of VA and PCA depend.

Standard methods of estimating urinary aromatic acids

(employing gas chromatography) have been developed by Williams (1962) and Williams (1965). Using these methods Williams and Leonard (1963) failed to detect PCA in the urine of children (at all events the level was below 0.5 μG/mgm creatinine). VA was present in two of four urines in amounts of 4.5 and 2.1 μG/mgm of creatinine. These methods employ diazomethane for methylation of phenolic groups and are unsatisfactory for detecting VRA in the presence of excess of VA and PCA.

A method has been developed by Dalgleish et al. (1966), in which both carboxylic and phenolic groups are converted into trimethylsilyl derivatives. Carter and Gaver (1967) produced an improved reagent which gives a complete reaction within 30 minutes and which has been used in the present study.

Horii et al. (1965) successfully separated the trimethylsilyl esters and ethers of the three compounds under consideration using silicone columns, and such a column was chosen for the present investigation.

Materials and Method:

The trimethylsilyl reaction was carried out using the following reagent : to 2 ml. of dry pyridine (redistilled and stored over KOH) were added 2.6 ml. of hexamethyldisilazane and 1.6 ml. trimethylchlorosilane. This admixture was carried out in a 20 ml. stoppered Exelo which had been allowed to stand overnight in a

desiccator, it being important to eliminate all traces of moisture. The solution was then centrifuged. The supernatant was clear and colourless and this constituted the reagent. It yielded satisfactory derivatives, known samples of PCA, VA and VRA each forming a single product.

The reagent was stored in the dark in a desiccator at room temperature and was used as required.

Standards were prepared either by using solid substances which were dried overnight in a vacuum desiccator at 44°C, or by evaporating known standard solutions made up in ethyl acetate and evaporated under a stream of nitrogen.

After adding a small amount of the trimethylchlorosilane reagent (50 - 100 μL) to the dried residue, the tubes were shaken vigorously and allowed to stand for 30 minutes at room temperature in a desiccator. 1 μL of the resulting solution was then applied to the gas chromatography apparatus.

The apparatus used was a Perkin Elmer Model F 11 gas chromatograph. The column used was a silicone Gum-Rubber E 301 on A.W. D.M.C.S. Chromosorb W 80 - 100 mesh ($1\frac{1}{2}$%), 1/8 inch in diameter and 2 meters in length. Nitrogen was used as the carrier gas. The temperature of the column was 128°C and the injection temperature 130°C.

If the retention of PCA in such a system is represented by

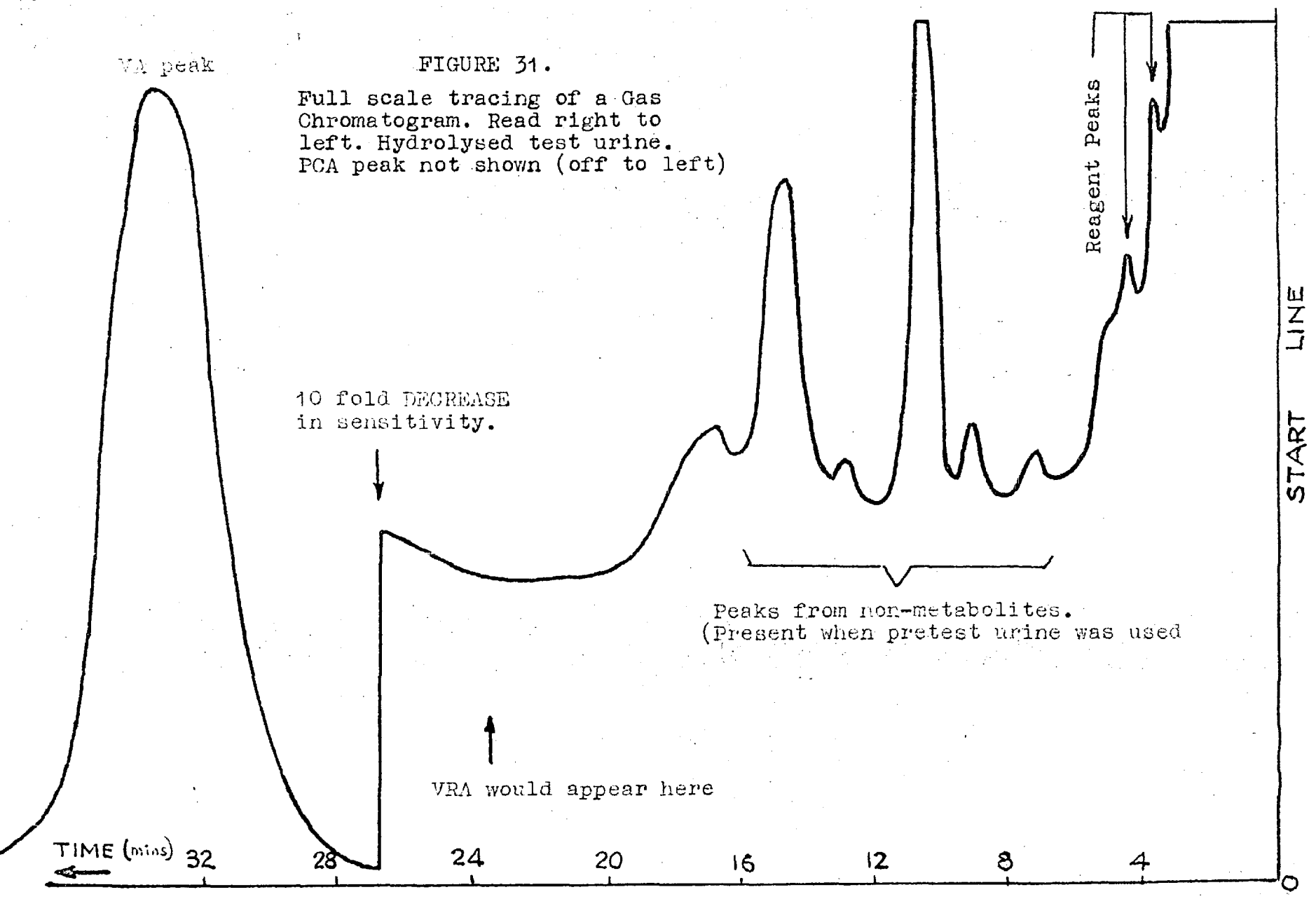


FIGURE 31.

Full scale tracing of a Gas Chromatogram. Read right to left. Hydrolysed test urine. PCA peak not shown (off to left)

1.00, that of VRA is 0.50 and of VA is 0.66.

Preliminary Findings:

If urine after PCA administration was acid-hydrolysed and an ethyl acetate extract prepared, evaporated to dryness, and the residue subjected to methylsilylation and injected under these conditions, the resulting gas chromatograph showed a characteristic appearance (see Fig. 31).

There was an initial peak due to the reagent, followed by a number of peaks and shoulders due to the presence of small amounts of phenolic acids which were not PCA metabolites (because pretest urine extracts similarly treated yielded similar peaks). No clear cut sharp peak likely to have resulted from VRA was detected. Several further peaks derived from non-metabolites appeared and then an enormous peak derived from VA, (authentic VA produced a peak with the same retention time as this enormous peak, no such peak being present when pretest extracts were used as starting material). After an interval a second large peak due to the methylsilyl derivative of PCA appeared (and could be identified as such by methods similar to those described for VA). The total run took 55 minutes.

IVA peaks and VA peaks did not appear separately using the conditions described.

c. VRA excretion after VRA administration. VRA is very insoluble

in water. It therefore seemed likely that any excretion of this compound would occur in the form of a VRA conjugate. That this was so was established by a test on a normal male subject who fasted overnight, collected a pretest urine & then took 500 mgm. of VRA by mouth. The subject thereafter collected urine for three hours, remaining fasting throughout this time.

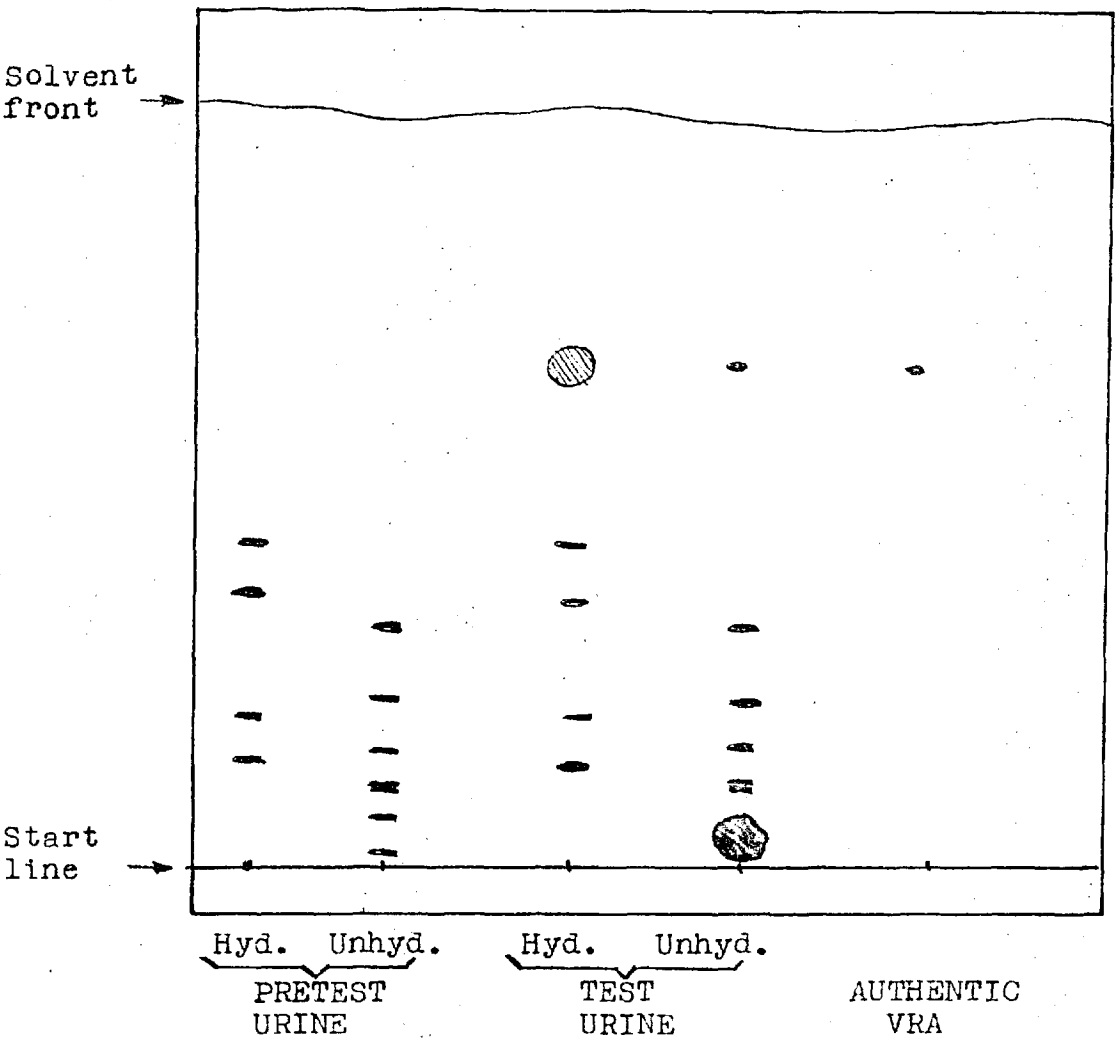
30 mls.of this urine were divided into two equal portions, both of which were subjected to acid hydrolysis under the conditions usually employed (15 lbs. per sq. inch for 1½ hours), and extracted in the usual way.

30 mls. of urine were extracted without preliminary hydrolysis as a control. Similar extracts were derived from hydrolysed and unhydrolysed pretest urine.

The ethyl acetate extracts were chromatographed simultaneously using the same solvent system (benzene, acetic acid, water : organic phase) and on similar T.L.C. plates to those used for VA/IVA ratio estimations. On this solvent system VRA runs distinctly faster than VA and IVA with clear separation. All applications were of the same size :- 2 μL. Authentic VRA was applied to the plate as a marker.

After chromatography and drying at room temperature, the plates were inspected under U.V. light (254 mμ), authentic VRA showing up as a dark area against the fluorescent background of the plate. A

Figure 32. TLC Plate showing the appearances obtained with urine extracts before and after the consumption of VRA.



Hyd.= Extract prepared from acid hydrolysed urine.

Unhyd.= Extract prepared from unhydrolysed urine.

Solvent System: Benzene : Acetic Acid : Water :: 2 : 2 : 1 (upper phase)

Plate impregnated with a fluorophor activated by 254 mμ light and viewed, unstained, under U.V. light.

small spot on the chromatogram of the extract derived from unhydrolysed test urine ran opposite authentic VRA, (no such spot being present on chromatograms derived from unhydrolysed pretest urine). A very large spot corresponding in position to authentic VRA was found onthe extract derived from hydrolysed test urine (no such spot being present on chromatograms derived from hydrolysed pretest urine). These results are depicted in Fig. 32.

Using methods identical with those described for identifying IVA, a U.V. spectrum of this material was obtained and shown to be identical with that of authentic VRA.

On thin layer chromatograms derived from unhydrolysed test urine, a large spot was seen near the origin (Rf. 0.05). This gave a positive stain with the Aroylglycine stain employed for V.A.G. identification. It seems likely that this spot (which was not present in chromatograms derived from the pretest unhydrolysed urine) was the glycine conjugate of VRA. The important point is that this spot had entirely disappeared in chromatograms derived from hydrolysed urine. These failed to show any staining with the aroylglycine reagent.

To summarise, these findings suggest that administered VRA is excreted largely as a glycine conjugate and that this conjugate breaks down completely under the conditions of acid hydrolysis employed routinely for the preparation of ethyl acetate extracts.

This is relevant to observations made on PCA test samples.

Thus, the failure to find VRA in the samples examined was due neither to a failure of authentic VRA to yield to the extraction procedures employed, nor was it likely to have been due to failure to hydrolyse a VRA conjugate.

Results. It became clear when a few extracts had been run that either VRA was not present at all, or that it was present in very small quantity. In order to accept VRA as being present, it seemed reasonable to lay down the following criteria, (which, however, do not amount to proof of the presence of VRA).

1. That a peak be present on the gas chromatogram having the same retention time as authentic VRA.

2. That the addition of the methylsilyl derivative of VRA to the material applied to the column for the first run increase the height of this peak.

3. That no peak fulfilling criteria 1 and 2 be present in chromatograms derived from pretest urine.

Normal subjects.

Twenty-seven specimens (acid hydrolysed PCA test urine) from normal subjects were examined, but because of limitations of time only one ethyl extract was prepared for gas chromatography from each subject.

Of the gas chromatograms obtained, 5 were technically unsatisfactory because the base line did not descend in a

satisfactory manner, 11 showed no trace of a peak in the VRA position, 5 showed very small peaks in the VRA area and 6 showed more definite peaks in the VRA area. Of the last mentioned, 4 peaks became distinctly larger when the authentic methylsilyl derivative of VRA was added but in no case was the pretest urine available for comparison. The 6 pretest specimens that were examined showed no clear peaks in the VRA position in any case.

Schizophrenics.

Eight samples derived from subjects 31 - 38 were examined. In each case a test and a pretest specimen were analysed.

One of the pretest samples showed a small peak at the VRA position. The other 7 showed no such peaks. Of the 7 that did not show such peaks, PCA test samples failed to show 'VRA' peaks in 6, the other chromatogram having an unsatisfactory contour in the VRA area. The other PCA test sample (deriving from PCA test urine from a subject (schizophrenic 35) with a peak in the VRA position in the pretest sample), also showed a peak in the VRA position in the test sample. The size of this peak was compared with the size of adjacent peaks derived from non-metabolites in both test and pretest specimens. This scrutiny did not suggest that the peak in the VRA position was relatively larger after PCA consumption.

It may be of interest to add that one subject who was a 20 year old youth with homocystinuria (described in detail elsewhere : Price et al., 1968) clearly met the criteria 1 - 3 stated above for VRA

identification after PCA testing. However, the PCA test in this subject gave normal results with respect to $\frac{\text{VA (total)}}{\text{PCA}}$ % and VA/IVA ratios.

Discussion:

The findings suggest that VRA does not appear to be a metabolite of PCA in normal or schizophrenic subjects, but this statement needs qualification.

1. The method was suitable for the detection of minute quantities of VRA but the starting material was not, because of interference from other carboxylic acids. (This situation could be improved upon by preliminary chromatography, a much purer fraction being used for the gas chromatography application).

2. In order to estimate the limit below which VRA would not be detected, a number of simple experiments were carried out.

Standard solutions containing VA/VRA ratios of 50 to 1 and 100 to 1 were made up in water and acid-hydrolysed under the usual conditions. Ethyl acetate extracts were prepared and these were evaporated to dryness, reacted and subjected to gas chromatography in the usual way. The chromatogram obtained where the ratio VA/VRA was 100 was compared with chromatograms obtained from test hydrolysed urine. The judgement was made as to whether or not, had the test extracts contained VA/VRA at a ratio of 100, the VRA peak would have been expected to be clearly visible, using the size of the VA

peak and the contour of the chromatogram in the VRA area as a basis for judgement (and making allowance for the change in the sensitivity setting illustrated in Fig. 31).

In all 19 of the control subjects in whom it was possible to make this judgement, it seemed likely that VA/VRA was greater than 100. This includes all 4 control subjects who showed peaks in the VRA position on the test samples. Of the 8 schizophrenics, the same judgement was made in all except No.35, in whom the ratio could have been as low as 1 in 50, but in this subject the pretest urine also showed a sizeable peak in the VRA position.

Conclusion:

1. A method has been described for separating PCA, VA and VRA using gas chromatography.

2. The ethyl acetate extracts used contained too many other organic acids to be an ideal starting material for this study.

3. If VRA was a metabolite of PCA, the amount excreted in the 3 hr. urine collection was small - less than 1% of the VA excreted, i.e. less than 0.5% of the administered dose of PCA.

This statement (which is tentative) applies to studies on 19 control subjects and 7 schizophrenics (including one (No.31) who showed the "pink spot" phenomenon at the time of the study, and one (No. 36) who had previously shown it).

4. It seems likely that any VRA that is excreted will occur as a

conjugate, probably a glycine conjugate.

A note on the absence of 3-hydroxybenzoic acid as a metabolite of PCA in man. Tompsett (1959), on paper chromatography evidence, states that 3-hydroxybenzoic acid is an important metabolite of PCA in man. The results of the present study do not support this. 3-hydroxybenzoic acid will be extracted by the procedures used in the preparation of extracts. It will form a volatile trimethylsilyl derivative (Dalgliesh et al., 1966). Dalgliesh et al. and Horii (1965) used silicone columns similar to the one used in the present study and both report that the derivative of authentic 3-hydroxybenzoic acid emerged earlier than those of VRA, VA and PCA which emerged in that order from their columns, as they did in the study presented here.

In the present study, pretest and test samples were, on a number of occasions, compared and by adjustment of the quantity of urine extract subjected to the methylsilylation process it was possible to obtain tracings in which the early peaks (due to urinary phenolics which were not PCA metabolites) were of equal size in samples derived from hydrolysed pretest and hydrolysed test urine, obtained from the same subject. It was often possible to superimpose the tracings and to match up test and pretest samples because of the similarity in contour of the early peaks, much as one might match finger prints. This could not have been done if

another metabolite was coming off the column in this early phase. The studies of Horii (1965) and Dalgliesh (1966) indicate that 3-hydroxybenzoic acid derivative would not come off the column so early as to invalidate this statement.

To summarise, the gas chromatography study provided no evidence to suggest 3-hydroxybenzoic acid as a metabolite of PCA in man.

The Protocatechuic Acid Test.

Dosage: Unlike the procedure adopted for the methacetin test, the dosage of PCA was directly proportional to body weight. A dose of 1 G. was given to a person of average weight (137 - 143 lbs.). A dosage schedule was constructed in which, as body weight increased, PCA dosage increased by increments of 50 mgms., so that for each dose of PCA there was a corresponding range of body weights. All persons falling within this range received the same dose.

Body weight and age: Defined as in the methacetin test procedure (p138 & 140).

The form of administration of PCA: The substance was administered in solution, this solution containing sufficient alkali to bring the pH to about 6. The solution was rendered more palatable by the addition of glucose, which had the additional property of acting as a reducing agent, protocatechuic acid being thus rendered less liable to oxidation.

The solution was not unpalatable to the taste nor offensive

Fig 33 PROTOCATECHUIC ACID TEST

designed to measure the rate at which methylation processes are carried out in the body

(Protocatechuic acid is a harmless chemical substance which has no pharmacological action: it is not classified as a drug. The 'acid' of the title will be neutralized before the substance is administered).

THE DAY BEFORE THE TEST

Ideally, the following should NOT be consumed; a note should be made of the necessary exceptions:-

All drugs, tablets and medicines.

All fruit(except that in jam).

Spinach, carrots, ice cream, custards, blancmange.

Coffee, cocoa, beer, coco-cola, fruit drinks.
(Tea and milk are permitted drinks).

SUBJECTS SHOULD FAST FROM 11 p.m.

THE DAY OF THE TEST

1. No breakfast should be taken, but water to quench thirst is permitted.
2. Subjects should not smoke on the day of the test until the test has been completed.
3. Subjects should empty the bladder just prior to administration of the substance. Enough of this urine to fill a universal container should be saved.
4. The substance used will be presented dissolved in a solution of dextrose, and is not unpalatable. The entire contents of the bottle should be seen to be consumed.
5. Subjects should fast thereafter for 1½ hrs, but should then take water freely. All urine passed in the 3 hrs. after the test should be collected in the container provided.
6. At 3 hours exactly after the time of consumption the subject should be encouraged to empty the bladder as completely as possible and this sample included in the container which has been provided.
7. The test should be completed before lunch is taken; the container should be kept in a cool place(i.e. a refrigerator) until collected.

to the eye, having the appearance of white wine and schizophrenics could invariably be coaxed into taking it. The volume given to a subject of average weight was 200 ml : about a glassful.

The solution was made up overnight for use the following day, being stored at 4°C. The making up of a solution eliminated the error which would occur if the solid compound were incompletely dissolved in a hot drink, and an undissolved residue left around the cup after drinking.

The instructions given to the technicians called upon to make up the test solution are given in Appendix 3 .

The protocatechuic acid test instructions: The precise instructions given to subjects undergoing this test are given in the accompanying figure (Fig. 33).

Fasting overnight ensured an empty stomach; breakfast was not permitted because it was deemed desirable to avoid differences in absorption attendant upon differences in gastric emptying which would occur with, for example, variation in the amount of fat consumed at breakfast time.

In paragraph 5 of the test proforma, the subjects are encouraged to refrain from drinking for 1½ hours after PCA consumption - this seemed a reasonable length of time during which absorption might occur, and during which the addition of a large water load to the intestinal contents with consequent drop in PCA concentration

would have been undesirable; afterwards free consumption of fluid seemed advisable so that the urine voided at the end of the three hours would be dilute rather than concentrated. Any error due to incomplete bladder emptying at this time would thus be kept to a minimum. This error is an unimportant one in a co-operative male subject for Vestergaard and Leverett (1958) have shown that after the bladder had been emptied voluntarily the amount of urine in the bladder on catheterisation was "no greater than what could be calculated to enter the bladder through the ureters during the time it took to do the catheterisation."

The urine collection.

The three hour period of urine collection was chosen as convenient, both for 'starved' medical student volunteers and for the supervision of difficult schizophrenics. During this three hour period, about three quarters (on average) of the administered dose was excreted into the urine (unaltered or in the form of metabolites). Collection of urine for a longer period would extend the period during which the research nurse would need to supervise the schizophrenic patients, without there being much likelihood that the conclusions drawn at the end of the study would be altered. Furthermore, errors due to extraneous phenolics become progressively larger as the collection period lengthens.

The urine collection was made into a container containing

0.2 ml. of 5% sodium azide. Strong mineral acid was not added because it was desired to estimate the pH of the urine lest this correlated with any of the measurements that were subsequently made. In retrospect it would have been wise to add a reducing agent also so as to eliminate the possibility of any oxidation of PCA that might otherwise occur.

At the end of the three hour period the healthy volunteers handed in their specimens; an aliquot was removed for pH estimation and 5 ml. 3N HCl added to the remaining specimen. With schizophrenic subjects (tested in mental hospitals some miles removed from the laboratory), the research nurse carried out the same procedures immediately the three hour test period had been completed. In the analysis of results the merit of examining urinary pH will be apparent : the excretion of one of the acids (unconjugated vanillic acid) was related to urinary pH.

The urine was processed for acid hydrolysis as soon after collection as was practicable and always within the first few hours, during which it was kept at $4^{o}C$.

Storage of urine.

An aliquot of the urine that remained was stored at $-20^{o}C$ for such further measurement or repeat measurement as should be required. Repeat estimations on four samples of urine thawed out after six months at $-20^{o}C$ showed no evidence of change in VA or

PCA content or in the VA/IVA ratio.

The replicability of the test.

The extent to which the various manoeuvres employed were successful in achieving a replicable test was put to the test (and found to be satisfactory) by a direct method : that of test/retest comparison (see p 269).

The selection of normal subjects for the PCA test. The method of obtaining volunteers was similar to that used for the methacetin test series (q.v.) and had the same limitations. A recruiting drive among nurses (aimed at increasing the proportion of females in the series) achieved only fair success; the final composition of the normal subject group was 42 males and 13 females.

The selection of acute schizophrenics for PCA testing. The considerations already discussed with reference to methacetin testing were applied also to PCA testing. A 64 year old patient who was included was an example of schizophrenia of late and acute onset; she was known to have a schizophrenic son. In addition, two patients diagnosed (by the late Professor F. J. Fish) as suffering from schizophrenia simplex were included.

A note on the reason that PCA testing was not applied to chronic mental hospital patients. There is a sizeable literature (which is well reviewed by Sourkes (1962)) debating whether or not there is a failure of glycine conjugation in chronic schizophrenia. Glycine

conjugation had been studied by administering benzoic acid and measuring the excretion of the glycine conjugate of benzoic acid, hippuric acid. No satisfactory explanation seems to exist for a) differences which some describe between chronic schizophrenics and non-schizophrenics and b) other investigators failing to find any such differences in similar studies.

Glycine conjugation enters into the PCA test. Failure of glycine conjugation could result in the formation of less VAG and less IVAG. Since VAG accounts for the majority of the methylated excretion, failure of glycine conjugation could readily mask any alteration in methylation and evaluation of results might well have proved indecisive or difficult in such a situation. It was considered that PCA testing of chronic patients would be an ill advised undertaking until the controversy about glycine conjugation has been settled by a direct method, namely the administration of benzoic acid, the metabolism of which is uncomplicated and therefore well suited to pharmacogenetic technique.

Glycine conjugation in acute schizophrenics has usually been reported as normal although Georgi et al. (1948) described marked impairment of hippuric acid excretion in early acute schizophrenics. In the present study no evidence of impairment of glycine conjugation in schizophrenia was found, but the amount of glycine required for conjugation was about 100 mgm., much less than in the

hippuric acid test in which several grams/glycine are required.

Measurements made on urine after PCA testing:

1. The following measurements were carried out on each urine specimen (55 normals, 21 schizophrenics):-

a) Volume : measured in a measuring cylinder at room temperature.

b) pH : on a Pye laboratory pH meter, to the nearest 0.1 of a unit.

c) Creatinine content : using the Folin method based upon the production of an orange red colour by the inter-action of creatinine with alkaline sodium picrate (King & Wootton, 1956).

d) PCA and 'total vanillic acid' (= T, see the next page), these values being derived from paper chromatography (Method 2(a)).

e) VA/IVA ratio (Method 2(e)).

2. In 30 normal and 21 schizophrenic subjects, the amount of urinary unconjugated vanillic acid was calculated (Method 2(b)).

3. In 27 normal subjects and 8 schizophrenics, an examination for the presence of VRA was carried out on the urine extracts, using gas chromatography (Method 3). The results of this study are included with Method 3 and will not be further considered here.

The method of calculating results, including a note on a necessary approximation. 1. If the values for vanillic acid were calculated entirely from the section on paper chromatography (Section 2a) they would be inaccurate because the 'VA' measured there included a small

amount of IVA.

When solutions of equal concentration of VA and IVA are made up in water, and colour developed using the Emerson reaction, the O.D. at 500 mμ of the solution of IVA is only 60% of that yielded by VA (personal observation). In section 2(a) the paper chromatographic technique used did not attempt to separate the two compounds.

If there are x mgm. VA and y mgm. IVA in a specimen of acid-hydrolyzed urine and if T is the 'total VA' derived from the method described in section 2(a) and if R is the ratio VA/IVA (section 2a), then

$$x + 0.6\,y = T$$

&

$$\frac{x}{y} = R$$

whence $x = T\left(\frac{R}{R + 0.6}\right)$ & $y = \frac{T}{R + 0.6}$

In the table of results, $\frac{x}{PCA}\,\% = \frac{VA(total)}{PCA}\,\%$ and

$$\frac{y}{PCA}\,\% = \frac{IVA(total)}{PCA}\,\%$$

2. No satisfactory method for estimating unconjugated isovanillic acid was found. After a 1000 mgm. dose of PCA the three hour urine collection seemed likely to contain about 2 mgm. of unconjugated IVA. At this low level interference from naturally occurring phenolics becomes substantial and accurate measurement becomes a considerable technical problem. Free

VA (section 2(b) above) presumably contains small amounts of free IVA but no data was available whereby exact values for the expression $\frac{VAG}{VA}$ and $\frac{IVAG}{IVA}$ could be derived.

The ratio used, $Log_{10} \frac{VAG}{VA(free)}$ is, therefore, an approximation which ignores the fact that $\frac{VAG}{VA}$ and $\frac{IVAG}{IVA}$ may but be identical. However, the ratio $\frac{IVAG}{IVA}$ was derived by the author (Price, 1969) for two normal male subjects who had consumed IVA by mouth. Values obtained for $Log_{10} \frac{IVAG}{IVA}$ were 0.89 and 1.06*. The mean value of $Log_{10} \frac{VAG}{VA(free)}$ in normal males in the present study was 0.919 (and their mean urinary pH was 6.0 : see footnote). This suggests that the amount of glycine conjugation undergone by IVA is of the same order as that of VA.

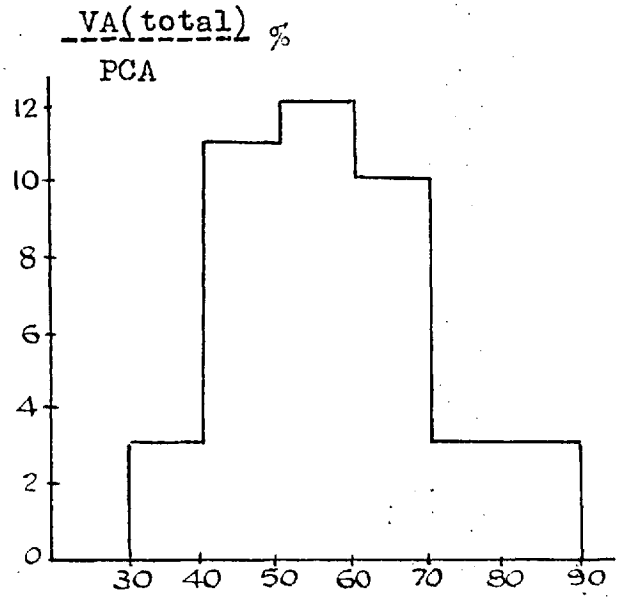
*pH of urine samples 6.1 and 6.0 respectively (see Fig. 41, p 265 in which a relationship between Log_{10} VAG/VA(free) and urinary pH is demonstrated graphically. As a similar relationship may hold between urinary pH and Log_{10} IVAG/IVA(free) it is necessary to specify urinary pH if a comparison between ratios is to be made).

Figure 34.

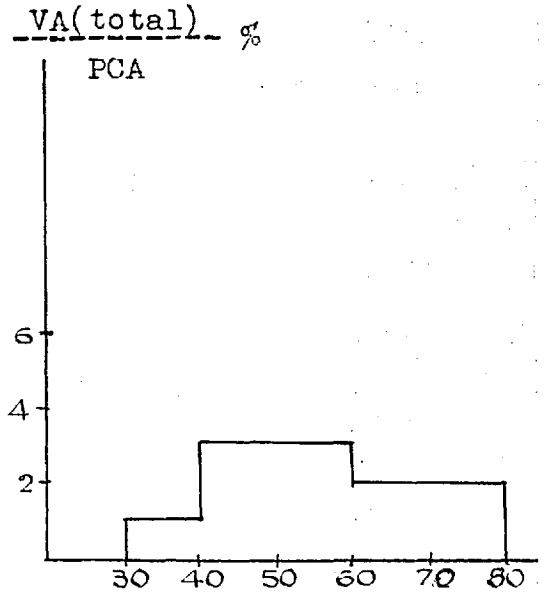
Histograms of $\frac{\text{VA(total)}}{\text{PCA}}$ % & $\frac{\text{IVA(total)}}{\text{PCA}}$ % for normal and schizophrenic male subjects.

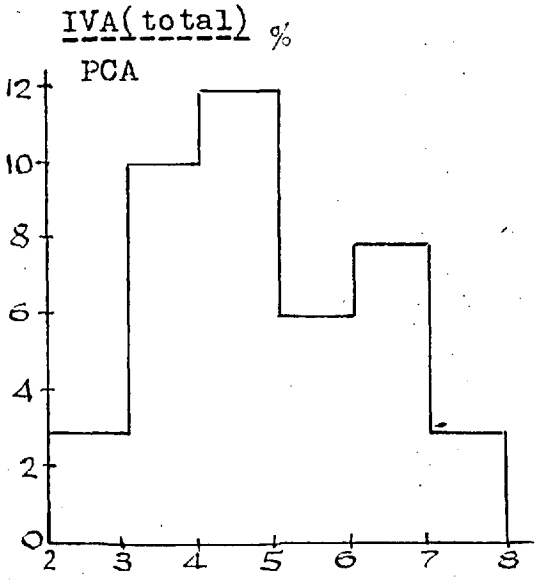
Figures on the ordinate represent number of subjects throughout.

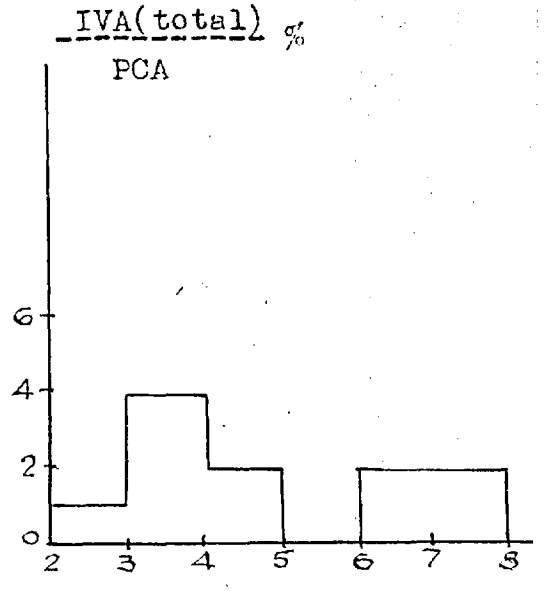
42 NORMAL MALES



11 SCHIZOPHRENIC MALES.







Results. a. Means, Standard Deviations and t-test comparisons between groups.

TABLE XVII. PCA Test, Means and S.D. for Males.

N.B. 'total' = free and conjugated.

	Control n = 42	Schizophrenics n = 11	't' test on difference between means t	p
Age (yrs)	21.0	28.7	4.02	<0.001
S.D.	3.4	10.7		
Body wt(lbs)	154.9	136.0	2.52	0.02>p>0.01
S.D.	20.0	29.1		
Vol (ml)	414.2	484.4	0.77	N.S.
S.D.	252.1	319.4		
pH	6.11	6.57	2.26	0.05>p>0.02
S.D.	0.60	0.71		
Creatinine (mgm)	215.19	239.0	0.84	N.S.
S.D.	43.17	167.0		
VA (total)/ PCA	57.5	52.6	1.09	N.S.
S.D.	13.12	14.00		
VA(total)/ IVA(total)	12.67	12.35	0.45	N.S.
S.D.	2.01	2.28		
IVA(total)/ PCA	4.70	4.66	0.07	N.S.
S.D.	1.36	1.81		
	n = 24	n = 11		
Log_{10} VAG/VA (free)	0.919	0.902	0.20	N.S.
S.D.	0.213	0.270		

Figure 35.

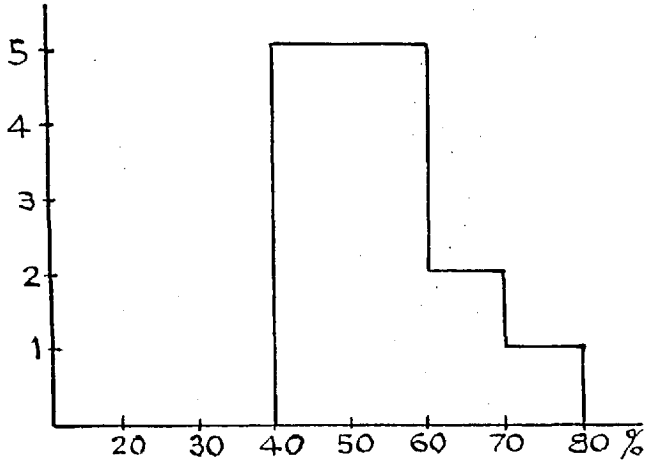
Histograms of $\frac{VA(total)}{PCA}$ % & $\frac{IVA(total)}{PCA}$ %

for female subjects.

Figures on the ordinate represent number of subjects throughout.

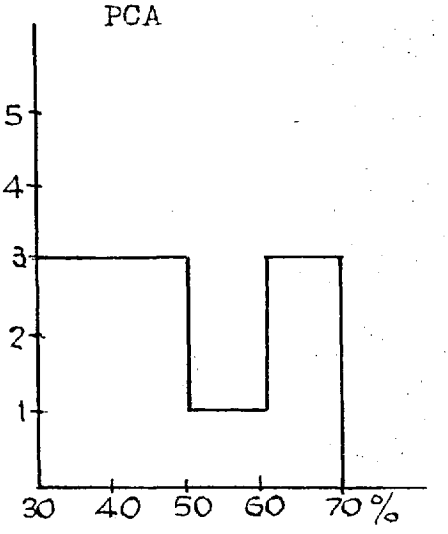
13 NORMAL FEMALES

$\frac{VA(total)}{PCA}$ %



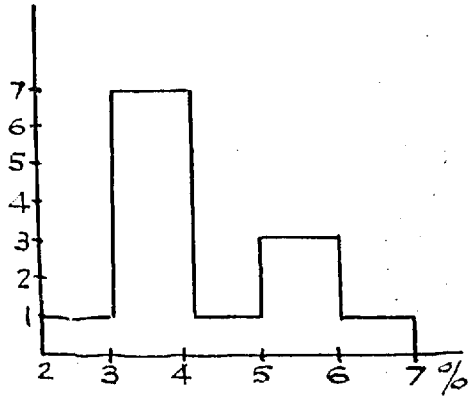
10 SCHIZOPHRENIC FEMALES.

$\frac{VA(total)}{PCA}$ %



All values of $\frac{VA(total)}{PCA}$ % are corrected for body weight.

$\frac{IVA(total)}{PCA}$ %



$\frac{IVA(total)}{PCA}$ %

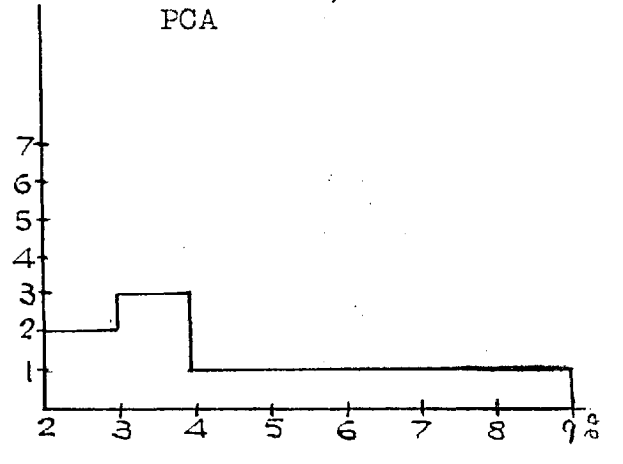
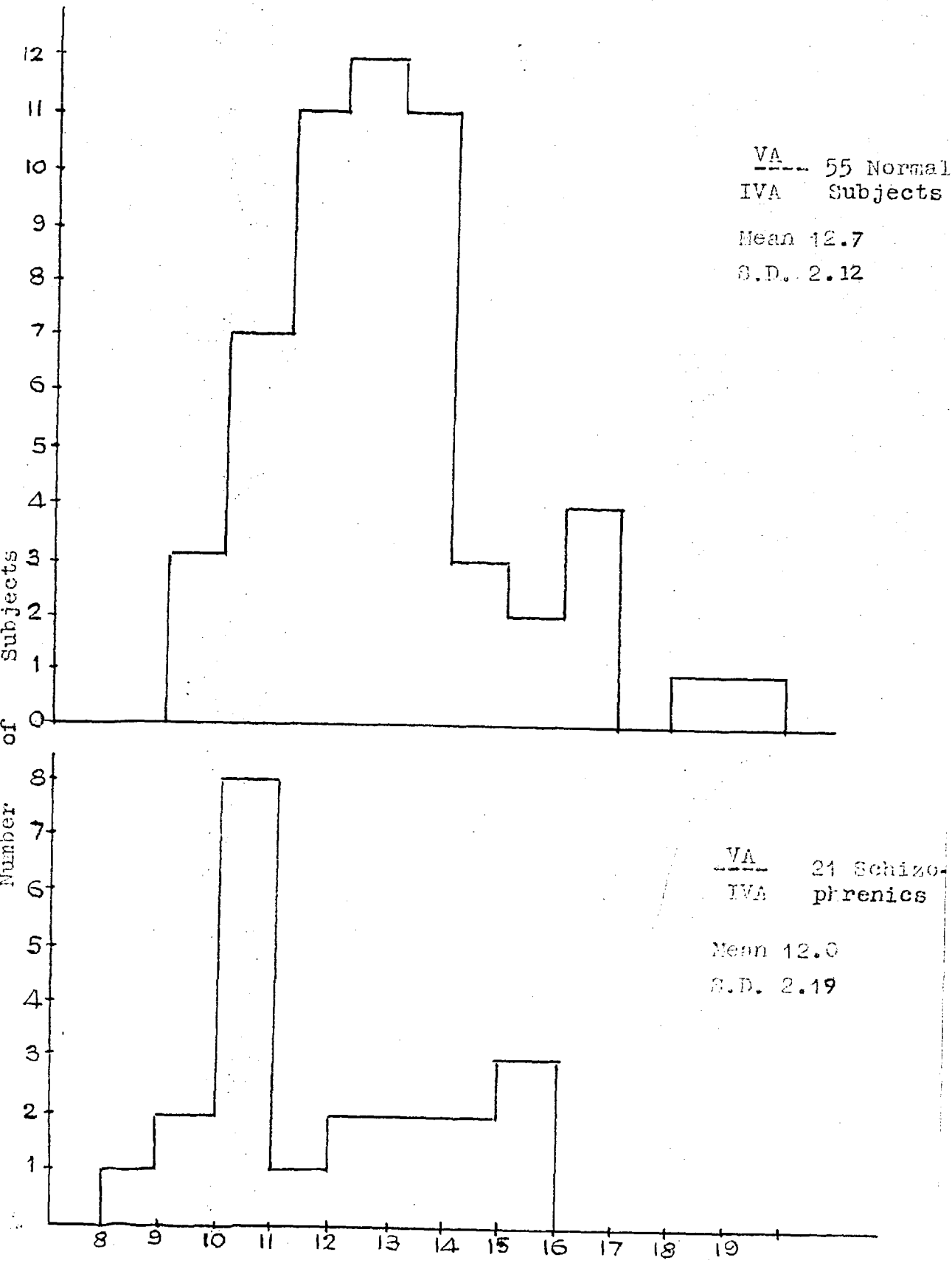


TABLE XVIII PCA test : Means and S.D. for Females.

N.B. 'total' = free and conjugated.

	Controls n = 13	Schizophrenics n = 10	't' test on difference between means t	p
Age (yrs)	23.8	33.5	5.44	<0.001
S.D.	5.5	12.5		
Body wt(lbs)	135.2	116.1	2.58	0.02>p>0.01
S.D.	13.7	21.7		
Vol (ml)	446.8	482.3	0.3	N.S.
S.D.	225.2	337.9		
pH	5.98	6.50	1.70	N.S.
S.D.	0.70	0.76		
Creatinine (mgm)	180.6	151.9	1.28	N.S.
S.D.	50.06	81.47		
VA(total)/ PCA%	51.4	55.4	0.60	N.S.
S.D.	11.94	20.29		
VA(total)/ IVA(total)	12.78	11.66	1.1	N.S.
S.D.	2.51	2.31		
IVA(total) PCA%	4.17	4.93	1.05	N.S.
S.D.	1.31	2.14		
	n = 6	n = 10		
Log_{10} VAG/VA (free)	0.921	1.017	0.60	N.S.
S.D.	0.239	0.339		

Figure 36 HISTOGRAMS of the $\frac{VA}{IVA}$ Ratio



Some of the results appearing in Tables XVII and XVIII are depicted graphically in Figs. 34 - 37.

There are no statistically significant differences in the methylation of PCA between schizophrenic and normal subjects.

Note on Fig. 35 concerning values of the ratio $\frac{IVA(total)}{PCA}$

Although values of the ratio for two of the schizophrenics lie above the normal range, these values (8.94 and 7.43) are not so high as to suggest that they indicate an important difference between these two subjects (Nos. 34 and 37 respectively) and normal females.

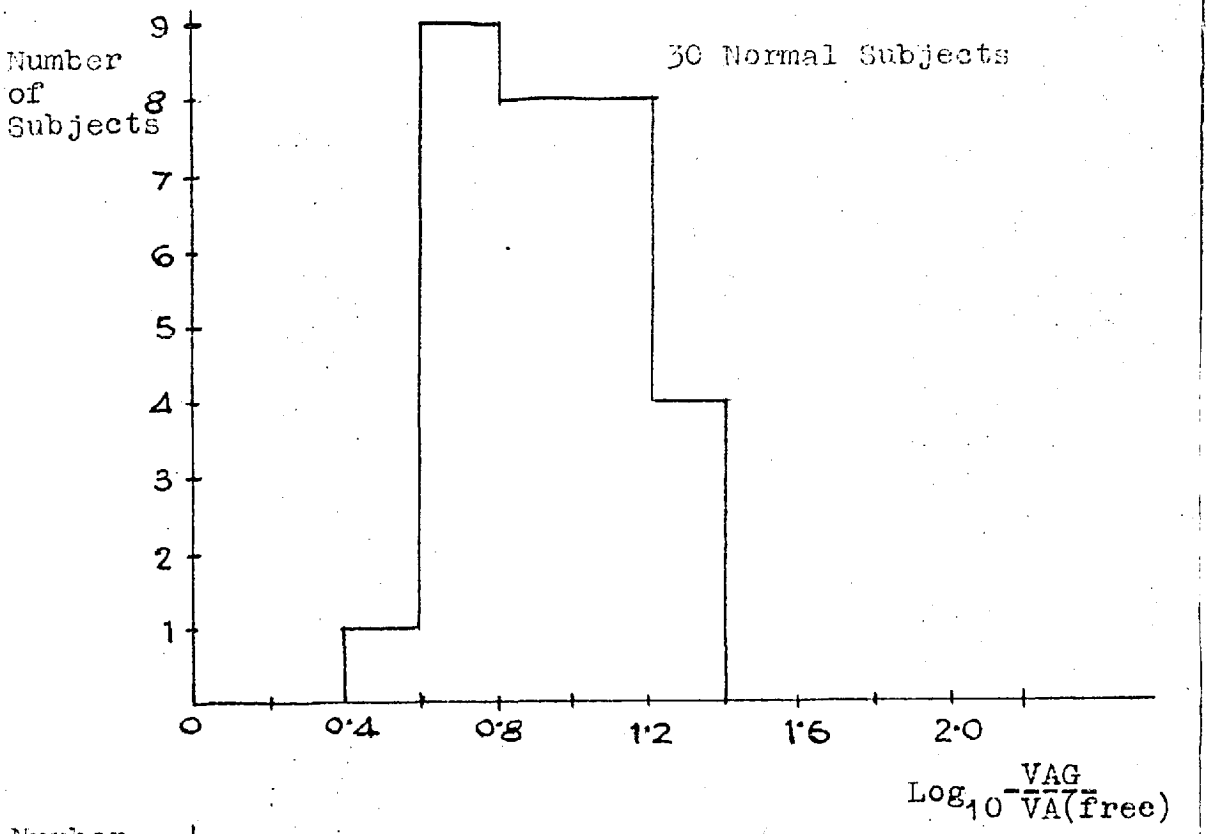
It may be noted from Tables 17/18 that the excretion of IVA does not differ significantly between schizophrenic and normal subjects, either in males or in females. It is worth noting in passing that if the PCA test were applied to subjects with amphetamine psychosis, results would not be relevant to the hypothesis of Smythies et al. (1967b) who suggested that "the psychosis found in amphetamine addiction might be associated with the production of 4-methoxy-amphetamine" (a potent hallucinogen in their view). This is because C.O.M.T. does not methylate monohydroxyphenols (e.g. p-hydroxy-amphetamine).

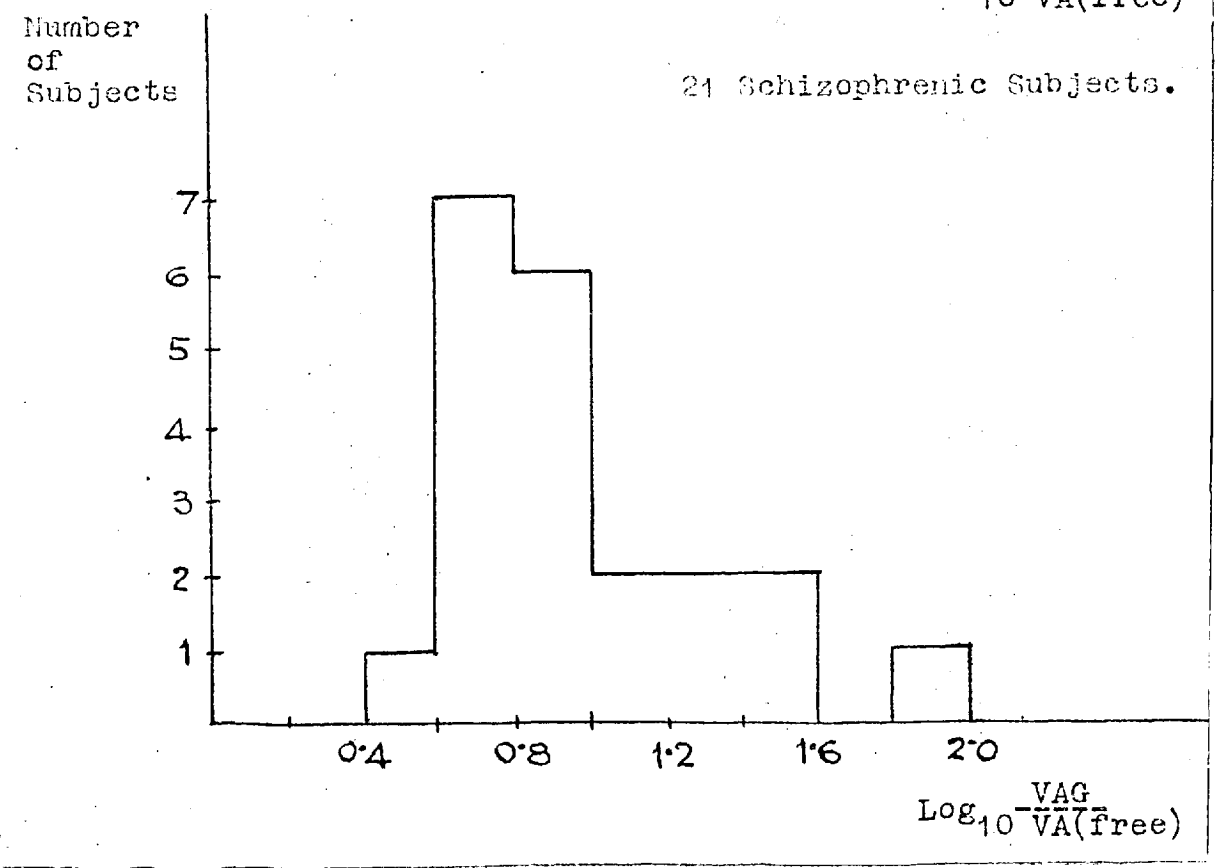
Comment on statistically significant differences between schizophrenic and control subjects shown in Tables XVII and XVIII

1. Age: In both male and female subjects, a statistical difference

Figure 37

The Glycine Conjugation of Vanillic Acid in Normal & Schizophrenic Subjects.





was found in age, the schizophrenics being older than the normals. This reflects the difficulty already discussed of obtaining volunteers outside the age range of university undergraduates and technicians (only the younger members of the latter group responding to the inducement of a modest financial reward).

2. Weight: Perusal of any tables on age and body weight in early and middle adult life indicates that, with increasing age, average body weight tends to increase. The findings that the schizophrenics (who were significantly older than the controls in this series) were significantly lighter than control subjects requires comment.

Rees (1943), in a comparative study confined to male subjects found that schizophrenics had significantly lower mean body weight than had control subjects. Betz (1942) reached a similar conclusion in a study confined to female subjects. Thus the present findings are in keeping with previous observations.

Differences in mean weight may relate to the suggestion (originated by Kretschmer) that schizophrenics tend to have leptosomatic body build. (In the present work no attempt was made to investigate somatotype and no measurement of a physical characteristic other than weight was made in the schizophrenic subjects). The difference might also be due in part to the tendency of

schizophrenic patients to lose weight as a result of their illness.

A further factor in the observed difference between schizophrenics and normals may be the above average physique of the latter. Thus, the average weight of the "normal" male subjects (average age 21) was 154.9 lbs. In the general population of males of 20 years of age Documenta Giegy (1959) give the average weight for persons of average height (5 ft. 8 ins.) and medium build as 143 lbs. As far as weight is concerned, one would judge that the control group (chiefly consisting of medical students) is unlikely to be a representative sample of the population.

To summarise, the observed difference in weight between normal and schizophrenics is probably due to a number of factors; previous studies of physical characteristics in schizophrenia do not make the observed difference a surprising one.

Urinary pH. Schizophrenic males had significantly higher urinary pH than did normal male subjects ($0.05 > p > 0.02$). The schizophrenics produced more urine than normals which may have contributed to a small extent to this effect, since dilute urine tends to be more neutral than concentrated (and more acid) urine. Otherwise the finding remains unexplained. The conditions of the PCA test include a number of special requirements prior to and after PCA administration. Other metabolic studies on schizophrenics do not contain such requirements prior to the measurement of urinary pH and are thus not comparable with the present study.

Results 2. Correlations within the groups of control and schizophrenic subjects.

TABLE XIX Control Group of Subjects : Correlations within the Groups for Males and Females Separately.

Data above and to the right is for males; below and to the left for females.

	Age	Body Wt	Vol.	pH	VA(tot)/PCA %	VA(tot)/IVA(tot)	IVA(tot)/PCA %	Log_{10} VAG/VA(free)
Age	-						*	
Body Wt.		-						
Vol.			-					
pH			***	-				**
VA(tot)/PCA %		*			-		****	
VA(tot)/IVA(tot)						-	****	
IVA(tot)/PCA %					****	**	-	
Log_{10} VAG/VA(free)				**				-

* = 0.05>p>0.02
** = 0.02>p>0.01
*** = 0.01>p>0.001
**** = p<0.001

N.B. Blank spaces in this table indicate that the value of p exceeded 0.05.

All correlations against urinary creatinine content were non-significant.

TABLE XX Schizophrenic group of subjects : correlations within the groups for Males and Females separately.

Data above and to the right is for males; below and to the left for females.

	Age	Body Wt	Vol.	pH	VA(tot)/PCA %	VA(tot)/IVA(tot)	IVA(tot)/PCA %	Log_{10} VAG/VA(free)
Age	-							
Body Wt		-						
Vol.			-					
pH			*	-				
VA(tot)/PCA %		*			-	***	****	
VA(tot)/IVA(tot)						-	****	
IVA(tot)/PCA %		*			****		-	
Log_{10} VAG/VA(free)				*				-

Key : as in the previous Table.

All correlations against urinary creatinine content were non-significant.

TABLE XXI Summary of those correlations shown in Tables XIX and XX in which a significant value of p was found for one or more groups of subjects.

No.	Correlation	42 male controls	13 female controls	11 schizophrenic males	10 schizophrenic females
1	pH v vol.	(+)	+++	(+)	+
2	$\frac{VA(total)}{PCA}_{\%}$ v body Wt	(-)	+	(-)	+
3	$\frac{VA(total)}{PCA}_{\%}$ v $\frac{IVA(total)}{PCA}_{\%}$	++++	++++	++++	++++
4	$\frac{VA(total)}{PCA}_{\%}$ v $\frac{VA(total)}{IVA(total)}$	(-)	(-)	---	(-)
5	$\frac{IVA(total)}{PCA}_{\%}$ v $\frac{VA(total)}{IVA(total)}$	----	--	----	(-)
6	Body Wt. v $\frac{IVA(total)}{PCA}_{\%}$	(-)	(+)	(-)	+
7	$Log_{10}\frac{VAG}{VA(free)}$ v pH	-- (see Note B)	--	(-)	-
8	Age v $\frac{IVA(total)}{PCA}_{\%}$	+	(-)	(+)	(-)

Note A. Key : 0.05>p>0.02 = + or -

0.02>p>0.01 = ++ or --

0.01>p>0.001 = +++ or ---

0.001>p = ++++ or ----

+ = positive correlation - = negative correlation.

(+) = non-significant positive correlation

(-) = non-significant negative correlation.

Note B. The ratio VAG/VA(free) was not normally distributed in any group studied (the most obvious example was to be found in the female schizophrenic group where the following figures were obtained : 9.15, 18.1, 6.29, 4.30, 6.15, 64.6, 9.34, 5.44, 11.8 and 12.8).

Conversion of each ratio to a Logarithm (to base 10), yielded histograms approximating more closely to normal distribution (see Fig. 37).

Values of Log_{10} VAG/VA were available for all schizophrenics and for 30 control subjects (24 males, 6 females).

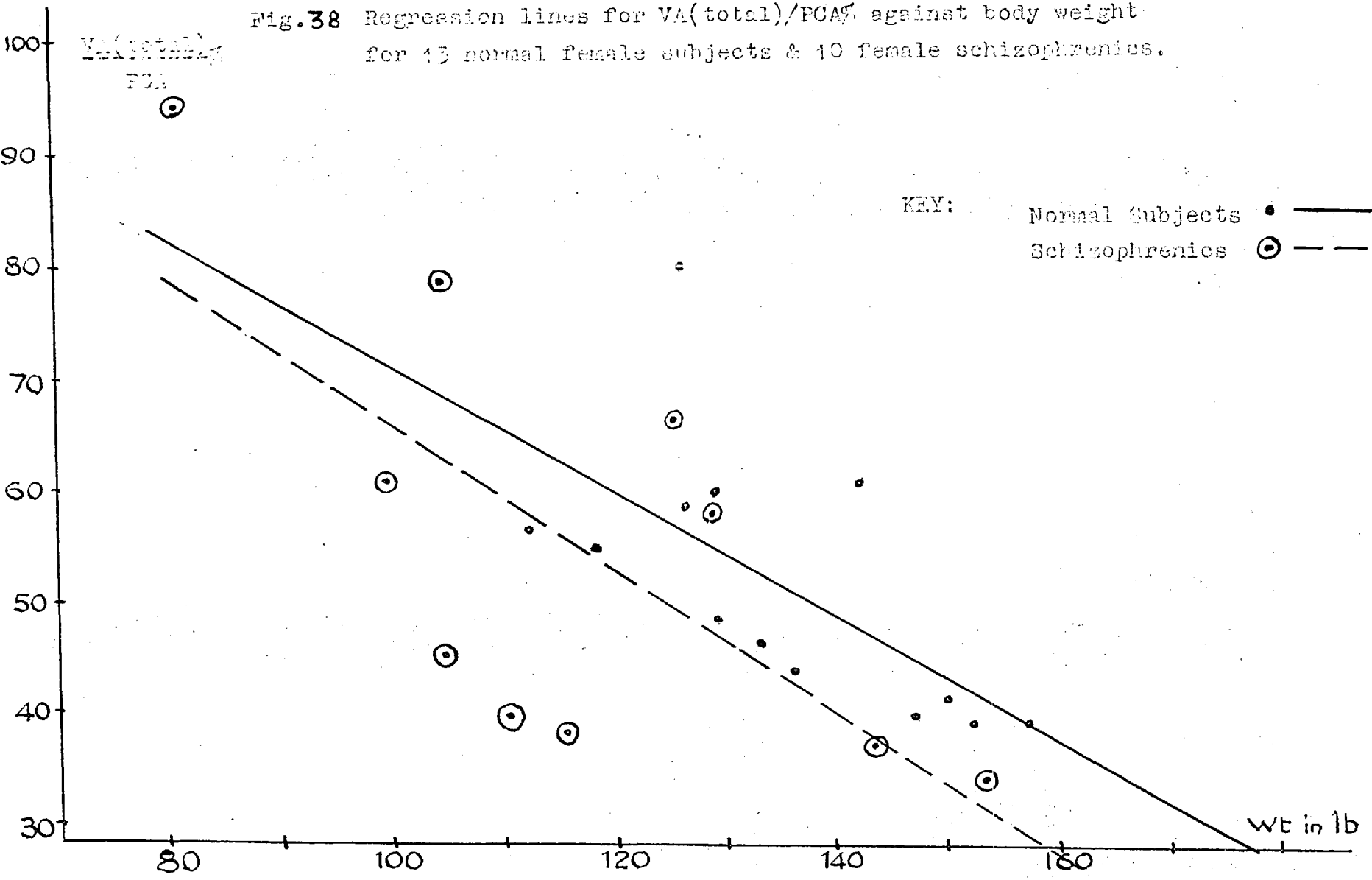
A systematic discussion of the observed correlations which have been summarised in Table XXI

All the statistically significant correlations are summarised in Table XXI . Inspection of this Table indicates that some correlations occur across the whole Table, whereas others do not. Some 'significant' correlations will be expected to occur by chance, a total of 112 correlations having been made in Tables XIX and XX

Each of the eight pairs of data giving statistically significant correlations will now be discussed individually to see what biological significance (if any) may be attached to them.

1. pH versus volume. Here a direct relationship was found in control and schizophrenic females. It probably reflects the fact that concentrated, small volume specimens of urine were usually

Fig.38 Regression lines for VA(total)/PCA% against body weight for 13 normal female subjects & 10 female schizophrenics.



more acid than were dilute, large volume specimens.

2. VA(total)/PCA % versus Body Wt. was a negative correlation in schizophrenic females with $r = -0.6867$ and $0.05 > p > 0.02$, a very similar state of affairs to that in female controls for whom $r = -0.6333$ and $0.05 > p > 0.02$. Taking all females, schizophrenic and non-schizophrenic, $r = -0.6424$, and whence $t = -3.841$ and $p < 0.001$.

If the regression line for y (VA(total)/PCA%) on x (weight in lbs.) is given by the formula $y = a + bx$, then 'b' is the slope of the line and 'a' the intercept on the y axis. These lines are, for the controls and schizophrenics respectively:

$$y = 125.76 - 0.551x \quad \text{and}$$

$$y = 130.02 - 0.642\,x \quad \text{(see Fig. 38).}$$

The weighted mean of the two gradients is -0.591.

The two lines do not differ significantly in slope ($t = 0.28$).

Analysis of variance of sums of squares of residuals from the regression lines gave the following (d.f. = degrees of freedom):-

	d.f.	Residual sums of squares	Mean squares	F ratio
Line for controls	11	1018.7552		
Line for schizophrenics	8	1958.5191		
	19	2977.2743	156.6986	2.2552
One line, for both groups	21	2270.4899		
Difference	2	706.7844	353.39	

The value of F for 2 and 19 d.f. is not significant at the 0.05 level.

This, together with the fact that the slopes of the lines do not differ significantly, indicates that the lines do not differ in position : the data could fairly be represented by a single line.

A correction can be made for the mean value of x (wt.) which was significantly greater (135.2 lbs.) in controls than in schizophrenics (116.1 lbs.).

This was done by using the formula

$$\bar{y} \rightarrow \bar{y} - \bar{\bar{b}} (\bar{x} - \bar{\bar{x}}).$$

where $\bar{\bar{b}}$ is the weighted mean of the two gradients and $\bar{\bar{x}}$ is the mean value of x for all 23 subjects ; $\bar{x}$ and $\bar{y}$ are mean values of x and y within each group.

Using this formula, the corrected value of $\bar{y}$, the mean for VA(total)/PCA% in controls becomes 56.2% and in schizophrenics 49.1%. Student's t test for the difference between the weight corrected means is not significant ($p > 0.1$).

The true difference between the means can be assigned 95% confidence limits of

$$(56.2 - 49.1) \pm (t) \times (\text{s.e. of difference}),$$

where t is obtained from a tabulation of t against p(0.05) and the number of degrees of freedom (21).

These limits are 17.5 to -3.35.

First order correlations using values of VA(total)/PCA% corrected for weight. Values for VA(total)/PCA% corrected for the effect of weight (using the formula $y \rightarrow y - \hat{b}(x - \bar{x})$) were obtained for all female subjects, and correlated against values of age, volume, pH, creatinine, VA/IVA, IVA(total)/PCA% and Log_{10} VAG/VA(free) similarly corrected for the effect of weight with the following results:

	Age	Vol.	pH	$\frac{VA(tot)}{IVA(tot)}$	$\frac{IVA(tot)}{PCA\ \%}$	$Log_{10}\frac{VAG}{VA(free)}$	Cr.
13 female controls	N.S.	N.S.	N.S.	N.S.	$p<0.001$	*N.S.	N.S.
10 schizophrenic females	N.S.	N.S.	N.S.	N.S.	$p<0.001$	N.S.	N.S.

* 6 normal females only. Cr. = Urinary creatinine.

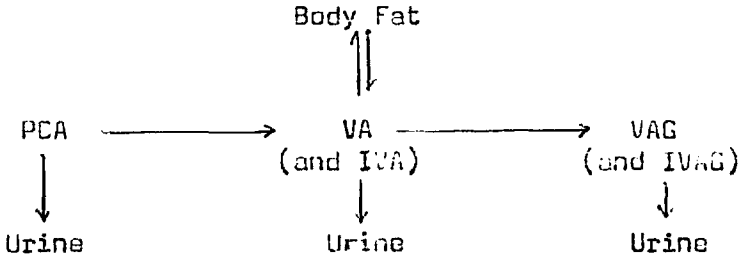
The dependence of the observed correlation on VA rather than on PCA excretion. It may be noted that, taking the female subjects as a whole, the correlation between $\frac{VA(total)}{dose}$ and body weight was significant ($r = -0.4388$, $t = 2.237$, $0.05>p>0.02$); that between $\frac{PCA}{dose}$ and body weight was not ($r = 0.2532$, $t = 1.199$, $p>0.2$). This suggests that the effect observed related primarily to vanillic acid, not to protocatechuic acid.

Discussion.

The reason for the correlation between VA/PCA and body weight for female subjects is not immediately obvious. One possible

explanation is that VA has an affinity for body fat (see Fig. 39).

Fig. 39 Schema for PCA metabolism.



Hence one would predict that increased body fat would mean less urinary VA and VAG and a lower VA(total)/PCA % value.

Evidence supporting such an explanation.

1. VA can be inferred to be lipid soluble by its behaviour in the renal tubule (v.i.).

2. Sex differences in the amount and more particularly in the variability of body fat gives some support to such an explanation.

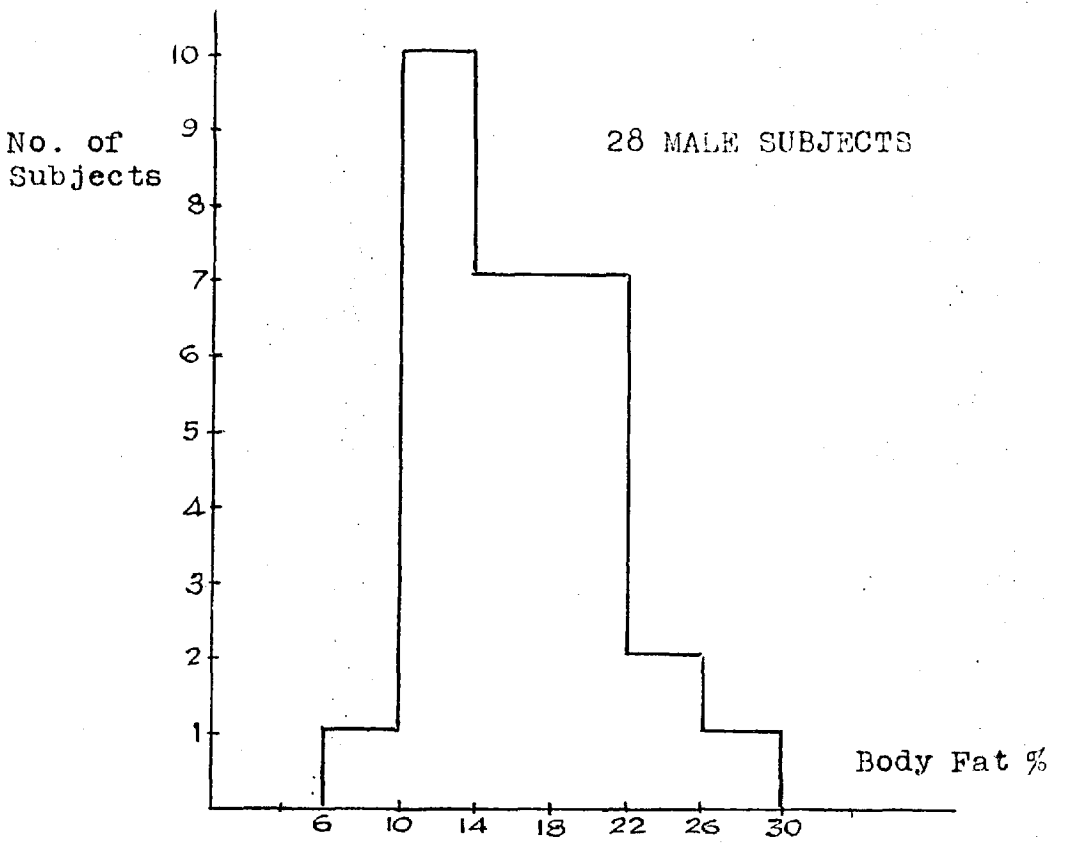
Thus, Grace and Goldrick (1969) provided formulae whereby body fat can be derived from height and weight. These were standardised on 17 - 45 year old subjects of both sexes (separately) (and are thus appropriate here). They have the following form:

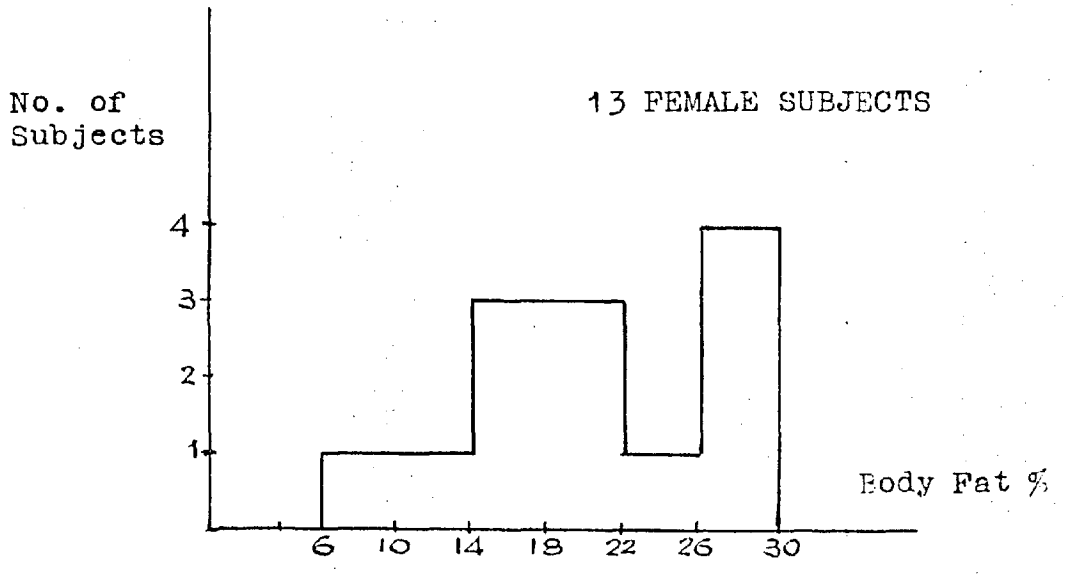
$$\text{Body fat} = \frac{K_1 \times \text{body weight}}{(\text{Height})^2} - K_2,\ K_1 \text{ and } K_2 \text{ being constants.}$$

Complete information on the heights of the schizophrenics was unobtainable (some being untraceable), but the formulae were applied to all female controls and two-thirds of the male controls (those readily available, some of the original group having left

Figure 40. HISTOGRAMS OF BODY FAT %

All subjects are control subjects. Percentages calculated by the method of Grace & Goldrick (1969).





the university at the time that knowledge of their height was required).

The values of body fat % thus obtained were as follows:-

	Mean		S.D.
28 male control subjects	16.21	±	4.71
13 female control subjects	20.69	±	6.25

Student's t test for the difference between the means gave a value for t of 2.544; with 39 degrees of freedom $0.02 > p > 0.01$, indicating significantly higher body fat in female subjects.*

This is in accordance with the findings of many other investigators (e.g. Anderson, 1963; MacMillan et al., 1965). What does not seem generally to have been found by others is the higher standard deviation in females for body fat %. Examination of the data graphically indicated that body fat % as determined in the 13 control female subjects did not approximate to normal distribution but was as shown in Fig. 40. This is presumably a chance happening related to the small number of subjects examined, and it might be expected to favour the occurrence of the observed correlation, should the explanation being offered here for this correlation be the correct one.

It must be admitted that the method used for estimating body fat is liable to considerable error; this would seem likely to be more important in males, for the formula used cannot make any

*Wilcoxon's test gave a z score of 2.2835 ($0.025 > p > 0.02$).

distinction between the contribution made to body weight by muscle and that made by fat. This might explain why a statistically significant correlation between the calculated values of body fat % and $\frac{VA(total)}{PCA}$ % was found in females ($r = -0.6006$, $t = 2.492$ $0.05>p>0.02$), but not in males where the correlation was not in the predicted direction ($r = 0.1314$, $t = 0.6758$, $p>0.2$).

The migration of chemical substances to body fat seems not to have received much attention, except in the case of volatile anaesthetics and pesticides. One exception is the drug pentothal, shown by Brodie et al. (1951) to be taken up to a substantial extent by body fat. Subsequently Brodie's conclusions have been questioned but Mark (1963) has discussed the problem and concluded that "uptake (of pentothal) by fat is neither the all-important parameter we erroneously conceived it to be originally, nor is it unimportant." For pentothal Mark quotes data indicating that pentothal uptake by body fat is fairly substantial (>38% at 1½ hrs.). However, it does not reach an early maximum, localisation in fat being greatest between 1½ and 2½ hrs; if vanillic acid behaves similarly, the duration of the PCA test (3 hrs.) may (inadvertently) have favoured the appearance of the observed correlation.

In the present study the evidence is not put forward as by any means conclusive, yet an alternative biological explanation of the observed results does not readily suggest itself.

3. VA(total)/PCA % versus IVA(total)/PCA %. A highly significant correlation appeared in all four groups ($p<0.001$ throughout). The most likely reasons for this would seem to be: a) that factors operating on unmethylated PCA (e.g. PCA absorption and PCA excretion) operated identically on both ratios and b) that the body handled VA and IVA once they had been formed in a very similar manner, (e.g. with respect to tissue distribution, excretion, glycine conjugation and the excretion of the glycine conjugate). (VA and IVA are isomers and have many physical and chemical properties in common). The only factor tending to prevent a high degree of correlation would appear to be the precise way in which methylation took place. This factor did not prove sufficient to prevent highly significant correlation occurring.

4. VA(total)/ IVA(total) versus VA(total)/ PCA % yields a statistically significant correlation for schizophrenic males ($r = -0.7487$, $t = -3.388$ and $0.01 > p > 0.001$.

Correlation coefficients for other groups of individuals are not significant.

Because, in females, the ratio $\frac{VA(total)}{PCA}$% correlates with body weight, weight corrected values of both $\frac{VA(total)}{PCA}$% and $\frac{VA(total)}{IVA(total)}$ were derived for females. In order that all data were treated alike, a correction ~~was~~ for weight was made in males, for both ratios.

When correlations were carried out on weight corrected values, the following data were obtained:-

	n	r	z	Corrected values of z	S.E. of z
Normal males	42	-0.155	-0.16	-0.16	0.16
Normal females	13	-0.244	-0.25	-0.24	0.316
Schizophrenic males	11	-0.727	-0.92	-0.88	0.354
Schizophrenic females.	10	-0.202	-0.20	-0.19	0.377

The mean value of z is $\frac{\Sigma(n-3)z}{\Sigma(n-3)} = \frac{-17.01}{64} = -0.2658$

This has a standard error of $\frac{1}{\sqrt{\Sigma(n-3)}} = \frac{1}{8}$

Testing the hypothesis that $\Sigma z = 0$ by means of

$t = \frac{\Sigma(n-3)\,z}{\sqrt{\Sigma(n-3)}}$ gives $t = \frac{17.01}{8} = 2.126$

For this, $0.05 > p > 0.02$, i.e. $\bar{z}$ differs significantly from zero. The correlation has, therefore, been examined using an analysis of covariance, as shown in Table XXII.

TABLE XXII Analysis of Covariance of the Correlation Between $\frac{VA(total)}{IVA}$ and $\frac{VA(total)}{PCA}$,

all values being weight corrected (separately for each sex), for the four groups.

This is laid out according to the method used by Snedecor (1956).

Line	Description	D.f	CSSX	CSPYx	CSSY	SLOPE	Df	Resid. SumSq.	Mean Sq.	F
1	Within group 1	41	7030.6	-162.076	156.239	-0.023	40	152.507		
2	" " 2	12	1023.5	-67.381	74.232	-0.066	11	69.796		
3	" " 3	10	1460.4	-196.458	49.996	-0.135	9	23.589		
4	" " 4	9	1971.3	-57.126	40.464	-0.029	8	38.809		
5	Total resid. within groups						68	284.601	4.187	1.270
6	Between internal gradients						3	15.950	5.317	
7	Common*		11493.8	483.041	320.931		71	300.631	4.234	1.228
8	Adj. means						3	15.603	5.201	
9	Total	75	12055.38	-417.911	330.721		75	330.721		

* Note: Common = Data on average of the gradients of the internal regression lines.

Conclusion: Neither value of F is significant; the 4 regression lines do not differ significantly in slope or position.

Comment on the Analysis of Covariance Shown in Table XXII.

It may be noted that the term VA(total) appears in the numerator of both the ratios being correlated. Any tendency which this has to influence the correlation should be in the direction of imposing a positive correlation. The observed relationship is in a negative direction, that is to say that there is a positive correlation between $\frac{VA(total)}{PCA}$ % and $\frac{IVA(total)}{VA\ (total)}$

Furthermore, $\frac{VA(total)}{PCA}$ % includes a contribution from $\frac{VA(total)}{IVA(total)}$.

If $R = \frac{VA(total)}{IVA(total)}$ then $VA(total)/PCA\ \% = T\left[\frac{R}{R + 0.6}\right] = \frac{T}{1 + \frac{0.6}{R}}$

(see p 243)

A high value of R would tend to produce a low value of $\frac{0.6}{R}$, a low value of $1 + \frac{0.6}{R}$, and a high value for VA(total)/PCA %. Hence the inverse relationship found occurs in spite of, not because of, the use of R in the computation of VA(total)/PCA %.

The relationship must be regarded as tentative for a very large number of correlations have been made, as previous Tables have shown, and p is significant at the 0.05 level, not below. Nevertheless, this particular relationship might be due to a biologically important phenomenon, and has been analysed in full for this reason.

Thus, one explanation that might be applied is that both ratios are dependent to some extent on the availability of methyl

groups, so that a relatively high availability of methyl groups leads to a high value for the $\frac{VA(total)}{PCA}$ % and to the formation of relatively more of the less readily formed isomer, isovanillic acid. The phrase 'availability of methyl groups' is not meant to imply availability from dietary sources. Had the latter been the important determining factor, the two ratios would have been unlikely to show the satisfactory degree of replicability that was found (see the replicability studies, p 269). Alternatively, speculation can be made that there might be two different enzymes (one concerned with 3-O, the other with 4-O methylation) these having different kinetic parameters.

5. IVA(total)/PCA % versus VA(total)/IVA(total). This negative correlation probably does no more than restate the fact that VA(total)/IVA(total) (=R, p 243) was used to compute IVA(total)/PCA %.

6. Body Wt. versus IVA(total)/PCA %. This correlation achieved statistical significance in the group of female schizophrenics only. It would seem likely that the body handles VA and IVA similarly (v.s.). Therefore the discussion concerning the relationship between VA(total)/PCA % and body weight may have relevance here (see p 254).

7. A striking correlation was noted between urinary pH and $\log_{10}$ VA/VA(free).

This is shown by the following data:-

	r	t	p
24 male controls	-0.488	-2.622	$0.02 > p > 0.01$
6 female controls	-0.887	-3.840	$0.02 > p > 0.01$
10 schizophrenic females	-0.652	-2.435	$0.05 > p > 0.02$
11 schizophrenic males	-0.304	-0.958	N.S.

It has previously been pointed out that a correlation existed between pH and urine volume (in both groups of female subjects). Data from the three groups (40 subjects in all) which showed a statistically significant correlation between $\log_{10}$ VAG/VA(free) and urinary pH were pooled and the following correlations obtained:-

		r	t	p
$\log_{10} \frac{VAG}{VA(free)}$	vs. pH	-0.4726	-3.3062	$0.01 > p > 0.001$
$\log_{10} \frac{VAG}{VA(free)}$	vs. Urine vol.	-0.3244	-2.1144	$0.05 > p > 0.02$
Urine vol.	vs. pH.	+0.4639	3.228	$0.01 > p > 0.001$

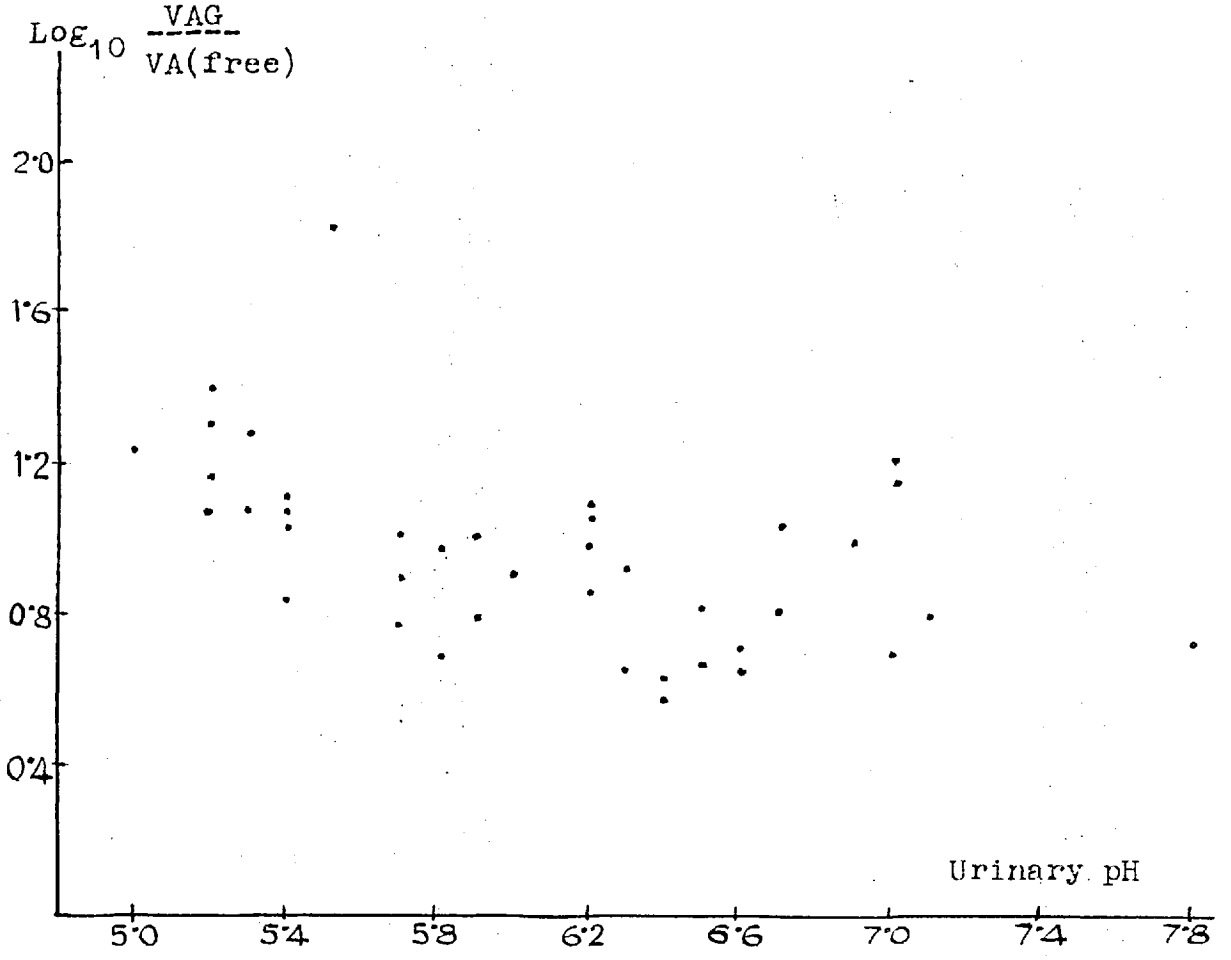
From this data, partial regression coefficients were worked out using the formula given by Tippett (1952).

This yielded the following correlations:-

a) between $\log_{10} \frac{VAG}{VA(free)}$ and pH, after adjustment for the effect of volume,

$r = -0.4588$ and $0.01 > p > 0.001$.

Figure 41. Plot of $\text{Log}_{10}\frac{\text{VAG}}{\text{VA(free)}}$ against Urinary pH for the 3 subject groups in which a significant correlation was found.



b) between $\text{Log}_{10} \frac{VAG}{VA(free)}$ and vol., after adjustment for the effect of pH,

$$r = -0.1929 \text{ and } p > 0.1$$

These calculations demonstrate that it is on urinary pH rather than urinary volume that $\log_{10} \frac{VAG}{VA(free)}$ depends.

Although values of $\log_{10} \frac{VAG}{VA(free)}$ could be corrected for the effect of pH by assuming a linear relationship, this has not been done. Such a linear relationship seems unlikely in view of the complexity of the physical chemistry which determines the effect, a complex subject which is of marginal relevance to the present thesis.

Fig. 41 shows the data graphically, and is worth the comment that pH appears to exert more effect on $\log_{10} \frac{VAG}{VA(free)}$ below pH 6 than it does above pH 6.

Since VA(total)/PCA % was not pH dependent (total VA consisting[5] largely of VAG), it seemed likely that what depended on pH was the amount of free VA excreted. In order to establish this, the ratios $\frac{VAG}{\text{total dose}}$ and $\frac{VA(free)}{\text{total dose}}$ were derived and the correlation coefficients between these ratios and urinary pH were determined for the 24 male controls. The same procedures were repeated on the data for all females (because of the small numbers all females were

grouped together).

The following results were obtained:-

		pH vs $\frac{VAG}{\text{total dose}}$	pH vs $\frac{VA(free)}{\text{Total dose}}$
24 normal males	r	-0.2089	0.6084
	t	-1.0021	3.5962
	p	>0.1	0.002>p>0.001
16 females	r	+0.3888	0.5955
	t	1.5793	2.773
	p	>0.1	0.02>p>0.01

From this one would conclude that it is the dependence of unconjugated vanillic acid excretion on pH which is responsible for the significant correlation between $\log_{10} \frac{VAG}{VA(free)}$ and urinary pH.

The finding that unconjugated vanillic acid excretion is directly related to urinary pH is not surprising. Milne et al. (1958) drew attention to the phenomenon of the non-ionic diffusion of weak acids and bases in a variety of sites in the body, including the renal tubule. Weiner and Mudge (1964) in a review of this phenomenon point out that, with a lipid-soluble compound which is weakly acidic, renal clearance is far greater in alkaline urine than in acid urine. For a pH dependent effect to occur, a sufficient degree of lipid solubility and a suitable pKa are necessary. Thus, while PCA and VA have pKa values which are very similar -

(4.48 and 4.52 respectively : Williams, 1959) only VA excretion depends on urinary pH.

Neither PCA nor vanillylglycine excretion depend on urinary pH : this is presumably because these substances are less lipid soluble, a fact which may be reflected in their low Rfs in the chloroform : glacial acetic acid : water system used in the estimation of VA and PCA, and illustrated in Fig. 26B p 202.

8. Age versus IVA(total)/PCA %. This appeared only in the group of normal male subjects, where the standard deviation for age was very small (3.4 yrs.). The most likely explanation of the finding (for which $0.05 > p > 0.02$) is that it is a chance occurrence. For the sake of completeness it may be noted that the correlation was a positive one, with $r = 0.3321$ and $t = 2.2271$.

Results 3. Recovery of metabolites.

Recovery was obtained by calculating the total amount of PCA, VA and IVA present in the urine specimen, and dividing by the dose administered. All values are slight overestimates, since no correction has been made for blank values.

	Mean	± S.D.
1. 42 normal males	74.02	11.28
2. 13 normal females	79.25	9.13
3. 11 schizophrenic males	73.76	13.95
4. 10 schizophrenic females	78.33	17.06

For differences between groups all values of Students' t give $p > 0.05$.

It will be noted that recovery for females was somewhat greater than for males. This was not what was expected, in view of the possible migration of vanillic acid to fat depots (which should be greater in females). Further analysis showed that the difference between recoveries was due to there being more PCA in the urine of females, recovery of methylated metabolites being almost exactly equal in the two sexes.

Mean values for the ratio $\frac{\text{PCA}}{\text{dose}}$ % for the groups are, respectively-

46.23, 51.24, 47.03 and 49.99.

Differences between the groups are not significant.

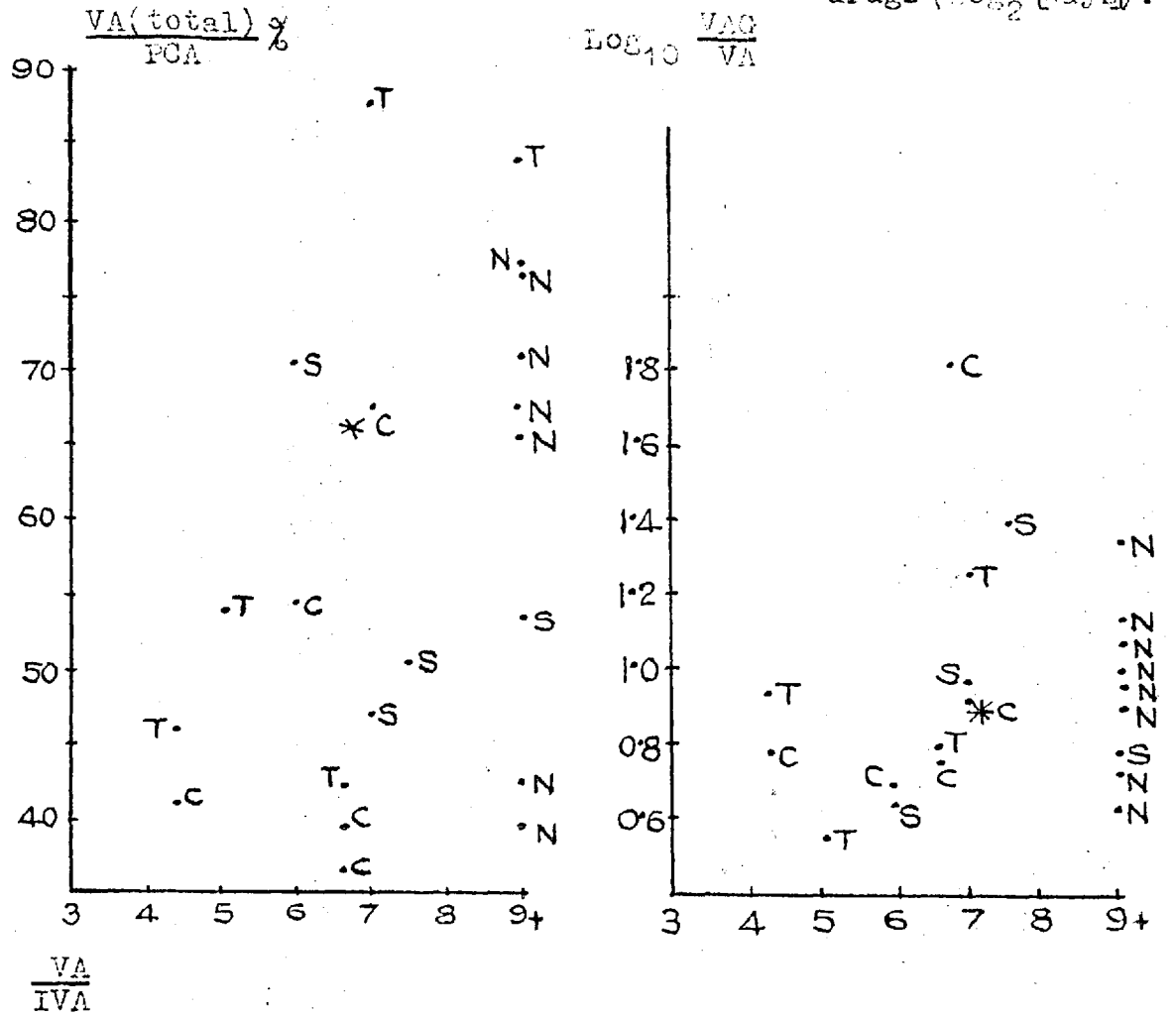
The close agreement between recoveries in control subjects and in schizophrenics suggests that cooperation by the patient and vigilance by the nursing staff were both of a good standard.

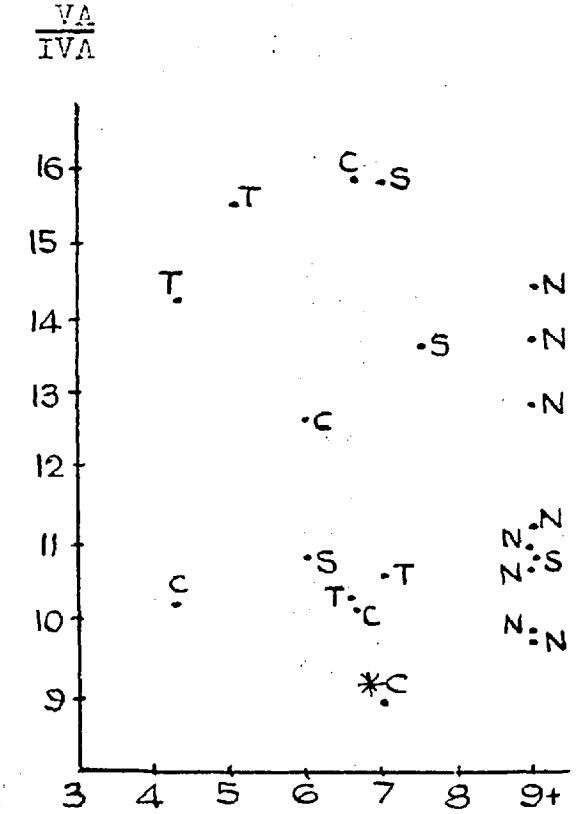
Results 4. The effects of phenothiazines on the metabolism of PCA.

Drugs presented less of a problem in this study than in studies with methacetin, because the length of time off drugs before testing tended to be longer and marked effects due to the phenothiazine medication seemed unlikely to occur.

The transformations that have to be considered are those of methylation and glycine conjugation.

Figure 42 PCA test results plotted against length of time off drugs ($\log_2$ [days]).





KEY. N = No phenothiazines.
T = Thioridazine.
S = Trifluoperazine, (Stelazine).
C = Chlorpromazine.

* This subject received a single dose of 10 mgm of Haloperidol on the day before the test.

As far as the activity of C.O.M.T. is concerned, Hertting et al., (1961) report that "chlorpromazine does not increase the activity of C.O.M.T. in vitro" (and presumably does not decrease it, although this is not stated). No reference has been found to the effects of phenothiazines on glycine conjugation. In Fig. 42 $\frac{VA\ (total)}{PCA}$ %, $\frac{VA}{IVA}$ and $\log_{10} \frac{VAG}{VA(free)}$ are plotted against length of time off drugs. No statistically significant correlations were demonstrated.

Results 5. The replicability of the PCA test.

Twelve subjects of the original control study (chosen largely from a conveniently available group of male medical students) were retested after about ten months. The dosage administered to subjects on the second occasion was determined from their weights at the time of the second test.

For each subject a new pair of values was obtained for the ratios $\frac{VA(total)}{PCA}$% and $\frac{IVA(total)}{PCA}$%. The ratio $\log_{10} \frac{VAG}{VA(free)}$, being dependent on urinary pH, was not derived in the replicability studies.

Results.

Subject No.	First Test VA(total)% / PCA	First Test IVA(total)% / PCA	Second Test VA(total)% / PCA	Second Test IVA(total)% / PCA
2	54.4	4.06	55.6	3.81
4	59.2	4.45	70.6	4.03
6	39.4	2.42	41.3	2.81
9	49.8	3.26	51.0	5.57
19	63.2	5.25	54.1	3.17
20	51.0	3.90	50.0	4.31
22	85.8	6.85	61.7	7.44
24	45.7	4.16	58.5	5.97
28	38.3	2.12	45.1	2.84
31	53.4	3.73	52.8	3.02
35	55.7	4.28	64.4	5.90
36	50.7	4.74	54.1	4.87

Results can be set out as an analysis of variance

a) for $\frac{VA(total)}{PCA}$ % values.

Source of Variation	Df.	Sum of Squares	Mean Sq.	F ratio
Between pairs	11	1871.80	170.16	3.717
Within pairs	12	549.38	45.78	

This value for F is significant at the 0.05 level.

The 95% confidence limits for the ratio of the variance components (i.e. the additional variance caused by taking members of different pairs divided by the 'within' pairs variance) are

0.0598 and 5.875

b) for $\frac{\text{IVA(total)}}{\text{PCA}}$ %

Source of variation	Df.	Sum of squares	Mean Sq.	F ratio
Between pairs	11	33.521	3.047	4.17
Within pairs	12	8.755	0.730	

This value for F is significant at the 0.025 level.

The 95% confidence limits for the ratio of the variance componants (as defined above) are 0.128 and 6.65.

Comment.

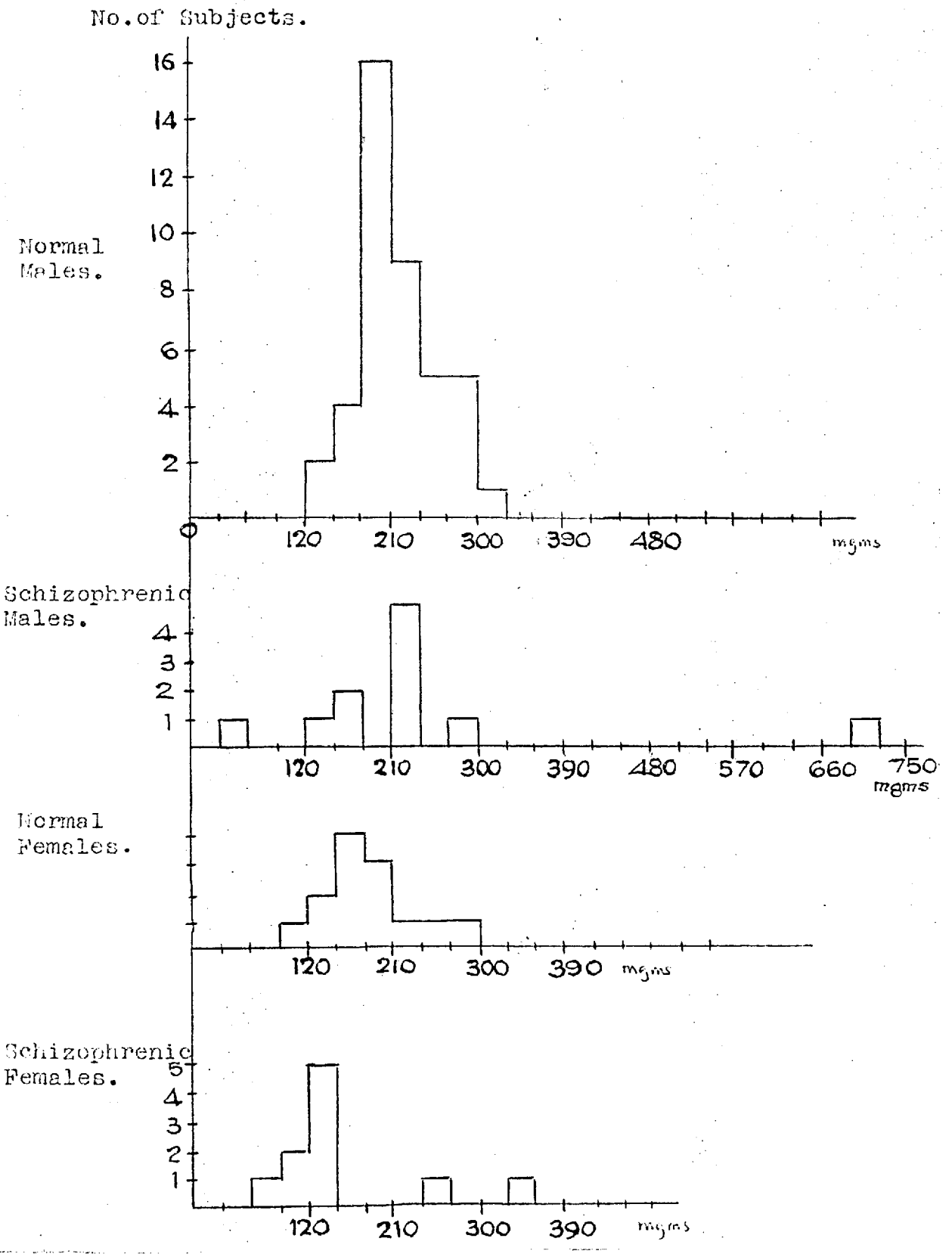
The results show that there was more variation in the two ratios between these subjects than there was between two results obtained from the same subject on different occasions and these findings indicate that the PCA test procedure yields a satisfactory degree of replicability.

Results 6. Creatinine Values.

The means and standard deviations (S.Ds.) for three hour urinary creatinine (to the nearest 0.1 mgm.) in control subjects and in schizophrenics were as follows:-

Figure 43

3 Hour Urinary Creatinines; Values obtained during PCA Test Collection Periods.



		Mean values	Standard deviation.
Control subjects	42 males	215.2	43.2
	13 females	180.6	50.1
Schizophrenics	11 males	239.0	167.0
	10 females	151.9	81.5

The important observation that can be made about this data is the higher standard deviations obtained for the schizophrenics. Two of the 21 schizophrenics had values above and three had values below the range for control subjects (see Fig. 43).

Factors influencing urinary creatinine. Vestergaard and Leverett (1958) showed that there was considerable variability between the creatinine values obtained in separate 24 hour collections of urine in single subjects, and comment that creatinine excretion should not be used for checking purposes in short urine collection periods. Values in shorter collection periods (2 hour periods in their study) showed greater variation than in 24 hour collections and they suggest tubular variability as the cause of this wide variation.

Doolan et al. (1962) showed that increasing dietary protein intake from 0.5 to 2.5 G/Kg. body weight led to a 10 - 15% increase in urinary creatinine; Bleiler and Schedl (1962) found a similar type of effect.

Bleiler and Schedl (1962) and Paterson (1967) found that exercise brought about little change in urinary creatinine. On the

other hand, Doolan et al. (1962) found that exercise slightly increased urinary creatinine.

Pscheidt et al. (1966) have studied creatinine excretion in schizophrenia (and in mental subnormality). They found a positive correlation between urinary creatinine and urinary tryptamine and comment that "sudden large elevations in urinary creatinine and tryptamine are at times, but not always, associated with a transient psychotic episode, preceded or accompanied by a refusal to eat proffered food and a modest loss in body weight."

They agree with Vestergaard and Leverett (1958), Paterson (1967) and Bleiler and Schedl (1962) that daily excretion of creatinine cannot be used as a basis for expressing metabolic data.

Gerard (1964), in an extensive study of schizophrenia, found urinary creatinine levels in schizophrenics to relate to reaction time, those with the faster reaction times having the higher creatinine levels.

Urinary Creatinine in the present study was estimated to see whether any light could be thrown on the low values obtained for urinary creatinine on the subject having abnormal methacetin test results.

One subject, E.G. (male, patient 31) produced a very informative result (and was also a 'pink spot' positive patient). This subject who weighed 10 st. 12 lbs. had a three hour urinary creatinine value of 55.4 mgms. This is 3.7 S.D.s below the mean

for 42 healthy male subjects. This subject excreted 75.9% of the administered dose of PCA in the three hours after the test (mean for 42 male controls - 74.02%). Clearly no gross omission in the three hour urine collection can have occurred.

The values obtained for urinary creatinine in the two four hour collections which yielded abnormal methacetin test results were 156 and 149 mgms. For a three hour collection these scale down to 117 and 111.75 mgms. respectively, values which are low when compared with values for normal subjects (2.3 and 2.4 S.D.s below the mean) but are not low when compared with the value for subject E.G. in whom the value for PCA recovery makes gross urine collection error highly improbable. To discard the methacetin test results M1 and M2 (see p 172) on subject P.B. as invalid because of subnormal creatinine content would seem, on this evidence, unjustifiable.

Urinary creatinine in other pink spot positive subjects. Three other patients were methacetin tested at the time that they were pink spot positive (Nos. 14, 16 and 22, p305-6). Four hour urinary creatinines on these three subjects' urine were 321, 219 and 208 mgm. respectively; scaled down to a three hour collection the values become 240.75, 164.25 and 156 mgms. respectively. Thus low urinary creatinines are not invariable in subjects showing the pink spot phenomenon. This is in accordance with observations made by

Dr. A. P. Ridges (personal communication).

High values for urinary creatinine in the present study. Two values from schizophrenics are worthy of comment : 339 mgm. for female patient 51 (D.K.) and 704 mgm. for male patient 45 (D.G.). These are 3.2 and 11.3 standard deviations respectively above the means for control subjects of the corresponding sex.

Part of the extremely high creatinine found in D.G. may be related to his considerable size (180 lbs; 6 ft. 1 in.), yet so great is the number of standard deviations of his creatinine level above the mean that this seems inadequate as the total explanation of the creatinine value obtained. Incomplete bladder emptying prior to testing might contribute to both high results, and cannot be excluded, although the routine of asking the patient to empty the bladder prior to PCA consumption was carried out and a protest specimen duly obtained from both D.K. and D.G.

Clinical Considerations.

The first subject (D.K.) was tested at a time when she was reported to be agitated and tense, deluded that she was going to be eaten by crickets, panicky and distractable. The second subject (D.G.) was reported at the time of testing to be anxious and agitated and to be suffering from severe persecutory delusions. He showed considerable fearfulness .

It is evident that both these patients were in a state of considerable agitation (because of the threatening nature of their delusions) at the time of testing.

Schottstaedt et al. (1956) in a study of the effects of emotional stress on creatinine excretion in normal subjects noted that the combination of one or more of the following : restless behaviour, apprehension, excitement, anxiety, uneasiness, anger or aggressiveness was associated with a rise in urinary creatinine above basal levels (although in their subjects only by about 10%). Sugard and Henry (1961) found a greater rise in urinary creatinine levels (and in 17 hydroxycorticoids) in airmen flying jet planes than in those flying other types of aircraft, and suggested that the former undergo more emotional stress.

Berlet et al. (1964) described a group of schizophrenics who showed the combination of high urinary creatinine, hyperactivity and aggression. Berlet's subjects also showed high rates of sodium and water excretion, and may correspond to schizophrenics described by Gerard (1964) who had fast reaction times, high urinary creatinine, increased urinary sodium and high urinary volume.

The high values for urinary creatinine in the two schizophrenics D.K. and D.G. would seem likely to be due to a metabolic response to a very threatening situation.

Low values for urinary creatinine obtained in three of the schizophrenics tested. Although three patients gave values for urinary creatinine which were outside the normal range, only one of these values, that for the patient No. 31 (E.G.) is remarkable : the other two low values were obtained in very small women, patients 34 (D.D.) and 50(D.P.) who were 5 st. 10 lbs. and 7 st. 1 lb. respectively.

The weight of male patient 31 (E.G.) was above average for the group, but his urinary creatinine lay 3.7 S.D.s below the mean for control male subjects.

This patient was, at the time of testing, described as dull, aimless, withdrawn, depressed and showing reduced activity. He was admitted in unusual circumstances, his mother coming down to the hospital with a report that he would not go to work and was spending his days in bed; a mental welfare officer then visited the house and arranged admission. The clinical features in this case were thus very different from the features in the two patients described above who had creatinine values higher than normal.

Schottstaedt et al. (1956) described a pattern of behaviour characterised by reduced activity and feelings of depression or exhaustion (in non-psychotic subjects) accompanied by retention of sodium, water and potassium and urinary creatinine values which were lower by an average of 9.6% than values obtained during

'tranquil' periods. The patient E.G. presented distinct resemblances to this 'low-activity' pattern of behaviour (and also had a low creatinine value in the three hour urine collection). A somewhat similar pattern of behaviour could be discerned also in P.B. (the subject with the abnormal methacetin test results).

Summary of observations on urinary creatinine in schizophrenics. Two examples of highly agitated apprehensive schizophrenics showing high creatinines in the three hour urine collection and one example of a low urinary creatinine in an inert, underactive patient were found in the present series and these findings are in accordance with (but may represent an exaggeration of) those described by Schottstaedt et al. (1956) as occurring with emotional changes of various types in normal subjects.

There would appear to be a need for further studies here, particularly over short periods of time, since it is the variability of creatinine excretion which is mentioned by several authors (e.g. Scrimshaw et al., 1966) as being characteristic during emotional stress; conventional 24 hour collections may mask important short term swings in creatinine excretion in schizophrenics. It would also be of interest to know precisely what symptoms in schizophrenics were important in influencing urinary creatinine levels, for it is clear that most of the schizophrenics excreted amounts of creatinine within the range for control subjects, and yet showed considerable clinical disturbance.

Discussion of the studies of in vivo methylation.

a) Comparison of methodology with that of previous studies. The present work differs from studies described in a previous section (p 41) in a number of important respects.

1. It did not concern itself with the methylation of amines, these being subject to destruction by amine oxidase so that methylation frequently becomes a relatively minor pathway. For the substrate used methylation was the major route of metabolism; because of the complexity of its metabolism, dopamine is a poor substrate with which to study methylation processes.

2. Previous studies have been concerned with the formation of D.M.P.E. and D.M.P.A.A. from dopamine. Work presented elsewhere by the author (Price, 1969) has shown that a pathway for the formation of 3:4 dimethoxy compounds in man does exist, since 3:4 dimethoxybenzoic acid is a metabolite both of vanillic and iso-vanillic acids. Thus the formation of 3:4 dimethoxy compounds can occur in two steps. Of these steps, methylation in the 4 position is the more interesting from the psychopharmacological point of view, and in the present study a separate quantitative assessment is made of this process. It is to be noted that Shulgin et al., (1969) in a study of the psychotomimetic properties of a large number of methylated one ring compounds in man (phenylethylamines, phenylethanolamines and phenylisopropylamines) make the point

that the conversion of a 3-OH group to a 3-OCH_3 group tends, on the whole, to decrease psychotomimetic activity. If this is correct, the study of 4-O-methylation becomes of more relevance to the proposition that the body might construct a psychotogen than does the study of 3:4 dimethylation.

3. Methodological objection can be raised to the work of Kuehl (1967) whose in vitro experiments on the conversion of dopamine to D.M.P.E. were carried on tissues collected between 3 hours and 6 days after death. The shortest period between death and autopsy for any of the schizophrenics from whom livers were obtained was 40 hours; most investigators would not regard conclusions drawn from the use of such necrotic tissue as having validity.

4. In the work of Friedhoff and Van Winkle (1963) only schizophrenic subjects were examined : there were no controls.

5. The scale of the present study is larger than that of previous comparative studies of methylation in schizophrenic and normal subjects, as shown by the following list:

	Control Subjects.	Schizophrenics	Method
Present study	55	21 mainly acute	In vivo
Kuehl (1967)	4	10	In vitro
Faurbye & Pind (1967)	1	4 chronic	In vivo
Friedhoff & Van Winkle (1963)	0	5 "confirmed diagnosis"	In vivo

The need for a sizeable survey in schizophrenia where aetiological homogeneity cannot be assumed has already been stressed.

6. The present study has, compared with that of Kuehl (1967), the disadvantage that a separate assessment of methylation by brain tissue was not carried out, nor was the material used to examine the dimethylation of the substrate a satisfactory one (see p 233). The present study is also open to the general objections which can be raised to the pharmacogenetic approach (see p 115 and 287).

b) Comparison of results between this study and previous studies as far as the faulty transmethylation hypothesis is concerned.

The present results are not at variance with the conclusions of other direct studies: with a catechol as substrate, it has not been shown that schizophrenics methylate differently from control subjects.

The results obtained draw attention to the need (which arises because methylated compounds may be lipid soluble) to study parameters which might easily be neglected. In the present study it was recognised that knowledge of urinary pH might be important for full analysis of results and it is worth noting that urinary pH could have been relevant in the present study even if analysis had been confined to an estimation of plasma levels, since the latter will be influenced (though not necessarily to an important

extent) by renal excretion. What was overlooked at the outset was that body fat might also prove a significant variable.

c) Implications of the present findings for future research.

(i) The possibility that vanillic acid has an affinity for body fat has already been discussed, and such an explanation of the data, if it is correct, is of particular interest because of its appearance in a study concerned with schizophrenia. This may, perhaps, best be put in the form of the single statement:-

1. that the distribution of a methylated compound may be related to body build.

This completes a series of statements (the basis for which has to some extent already been discussed):-

2. That some methylated compounds possess hallucinogenic activity.

3. That some resemblances exist between hallucinogenic states induced by drugs and schizophrenia.

4. That schizophrenia is related to body somatotype, being uncommon in those of pyknic build (Slater & Roth, 1969).

One question which comes to mind is whether body fat could contain biologically important quantities of amines. The dependence of vanillic acid excretion on urinary pH is due (in part) to the lipid solubility of vanillic acid. Another compound the urinary excretion of which is pH dependent is tryptamine (J.A.G. Watt, personal communication). Does tryptamine (which

is of particular interest because its excretion has been reported to increase during exacerbations of schizophrenia) have an affinity for body fat?. What other amines do? Does sudden fat breakdown release amines (including tryptamine) and could this relate to Kallmann's observation (1953) that a drastic reducing diet tended to have an adverse effect on the clinical state in schizophrenia? Does the body fat of the schizophrenic contain any amines not present in fat from non-schizophrenic subjects? Are any of these causally important in the disease? If the production of methylated (and therefore possibly lipid soluble) amines is related to the occurrence of the symptoms of acute schizophrenia, the obese subject might be at an advantage in having a larger reservoir of fat. "Which MZ twin was the fattest?" could be a relevant question in a study of twin pairs discordant for schizophrenia.

Research directed at answering some of the questions posed above would seem both worthwhile and practicable.

(ii) The present findings of an inverse relationship between $\frac{VA(total)}{PCA}$ and $\frac{VA}{IVA}$ would be worth re-investigating by in vitro techniques, where a less tentative result might be anticipated.

(iii) Further experiments into the correlation between creatinine excretion and clinical symptomatology would appear to be indicated. The correlations in the present thesis have been made retrospectively

and are speculative. There may be something to be gained by carrying out short term collections rather than the more conventional 24-hour collections (this is discussed above, p 278).

A note on the content and conclusions of this thesis.

The present regulations of the University of London governing the degree of Doctor of Medicine require the candidate to state clearly "what he considers to be the claims to originality of the thesis as a contribution to the science or practice of medicine" and to indicate in what way he advances "medical knowledge and practice."

The studies presented here are of an original nature : they do not attempt to replicate earlier work by others.

They suggest (as far as the compounds studied are concerned) that the demethylation and methylation processes involved do not generally differ between schizophrenics and non-schizophrenics. The detailed study of PCA metabolism includes new and reasonably satisfactory methodology for estimating the various metabolites studied and throws light on the mechanism of methylation in man and on some factors influencing the distribution and excretion of a methylated compound which may have applications elsewhere.

The thesis also provides an example of a relatively new science (pharmacogenetics) being applied to a long-standing clinical problem.

SOME PRACTICAL IMPLICATIONS OF THE PHARMACOGENETIC APPROACH.

A. The choice of chemical substances for pharmacogenetic experiments with particular reference to methacetin and protocatechuic acid.

Certain criteria for the selection of substances for the type of experimentation which is presented here deserve attention. These are summarised in Table XXIII (the numbers of which are used in the discussion which follows). Some of the information in this Table is dealt with at length elsewhere (as is indicated by page references).

1. Harmlessness and 2. Clinical effects. In administering tests to schizophrenics where no direct benefit in terms of therapeutic response or increased clinical understanding can be claimed to result from the test, any procedure which might cause harm is ethically unacceptable. Toxic substances, or substances responsible for drug reactions of a non-toxic type or substances with powerful pharmacological effects which are not beneficial, are thus debarred from use.

3. Purity. For metabolic studies, a very high degree of chemical purity is advisable, not only because of analytical difficulties which may arise when mixtures of chemical substances are given, but also because impurities may have toxic properties.

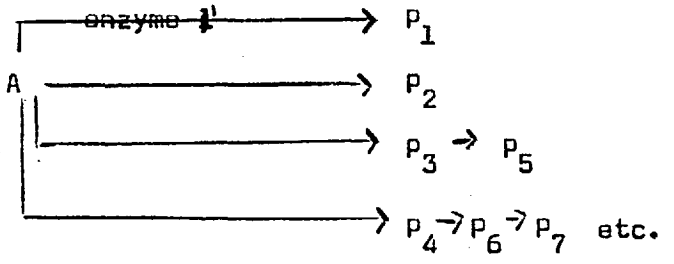
TABLE XXIII Summary of the suitability of the methacetin and PCA tests for the experiments in which they were used.

	Methacetin	PCA
1. Harmlessness	A single dose probably as harmless as a single dose of phenacetin.	No evidence of toxicity in the dosage given
2. Clinical effects	Analgesic and antipyretic	No obvious effects in dosage given
3. Purity and availability	Obtainable in crystalline form (British Drug Houses)	Satisfactory purity when obtained from SIGMA Chem. Co.
4. Average dose	500 mgm.	1000 mgm.
5. Cost of average dose	1d.	1/2d.
6. Metabolism.	O-demethylation Subsequent conjugation Paracetamol available to check the conjugation stages if test abnormality found	3 & 4-O-methylation. Then glycine conjugation. 5 metabolites (possibly 6) (including unaltered PCA).
7. Chemical analysis	Easy	Cumbersome
8. Suitability as judged by control group results.	Suitable	Suitable
9. Sensitive to small changes in rates of demethylation or methylation.	Probably insensitive (see kinetic studies, p 128).	No obvious reason why the test should not be sensitive.
10. Replicable	Yes	Yes
11. Rate of clearance from body.	Rapid	Very rapid
12. Stability of drug and metabolites.	No evidence of instability.	Metabolites of PCA stable. PCA stable in the presence of mineral acid or reducing agents.

4. The amount of chemical substance to be administered. There are distinct advantages in administering substances (unless they carry a radioactive label) at a 1 G. level rather than at a 1 mgm. level. Interference in chemical estimation is more likely to be troublesome when working at low dosage levels, particularly in schiz phrenics who will often have been taking a variety of drugs.

5. Cost. This should not be prohibitively high.

6. Metabolism. Here one is on difficult ground because of the complexity of the subject. Such factors as rate of absorption, completeness of absorption, distribution in body fluids, metabolic pathways and their kinetics and clearance of metabolic products all play a part in determining what is excreted and by what route, and in what quantity. But even in this complex situation certain criteria can be formulated; thus, a drug which has a large number of metabolites is generally unsuitable for study. Suppose a drug A has products P_1, P_2, P_3 P_4 etc.



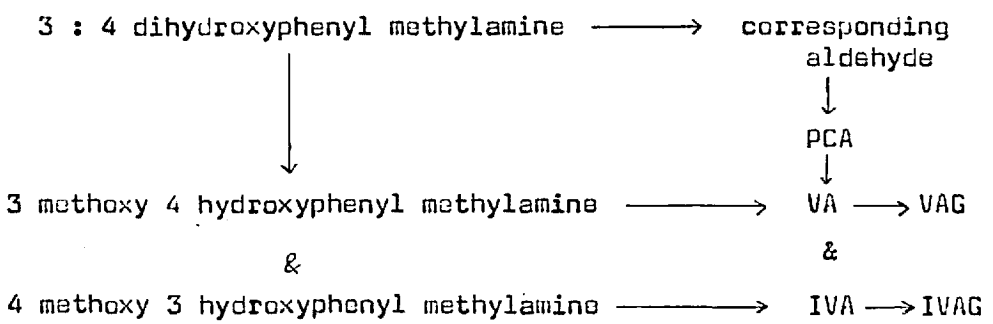
In this situation, to study enzyme 1, by estimation of A and P_1 is likely to be uninformative, (unless $A \rightarrow P_1$ is the major pathway) because, if enzyme 1 were reduced to half quantity, the difference this made to P_1 production could well be masked by the activities of enzymes 2, 3, 4 etc. responsible for $A \rightarrow P_2$, $A \rightarrow P_3$ $A \rightarrow P_4$ etc. A simple metabolic transformation, where the major pathway for the substance administered is the pathway one wishes to study is what the investigator should be looking for. Unfortunately, there may be no drug which satisfies ideal requirements. Thus in a study of O-demethylation, whatever the initial compound administered, one is likely to have not the single reaction $R - OCH_3 \rightarrow R - OH$, but a set of reactions - $R - OCH_3 \rightarrow ROH \rightarrow$ R-O-sulphate or R-O-glucuronide because the phenolic group formed by demethylation is frequently dealt with by the formation of such conjugates. This is the situation which complicates a study of methacetin metabolism. Here, however, R-OH equals paracetamol. Thus, suppose that the stage $R-OCH_3 \rightarrow R-OH$ proceeds normally, but a fault occurs thereafter (as may occur in Gilbert's disease in which paracetamol conjugation with glucuronide is faulty, Axelrod et al., 1957). Then not only will the metabolism of methacetin be interfered with, but also that of paracetamol. On the other hand, if demethylation only is at fault and subsequent metabolism (paracetamol conjugation and the excretion of

conjugates) is normal, such a situation will be recognisable by normal paracetamol metabolism in the presence of faulty methacetin metabolism. This exemplifies a resource available to the investigator who used the pharmacogenetic approach.

Turning now to the choice of protocatechuic acid, several important points need to be made:-

a) The 3 : 4 dihydroxy configuration is a configuration which occurs in several important neurochemical transmitters namely dopamine, adrenaline and noradrenaline. Choosing a substance such as 3 : 4 dihydroxybenzoic acid (PCA) allows one to study not only the extremely well known process of 3-O-methylation but also the less commonly studied process of 4-O-methylation.

b) By using an acid instead of an amine or an aldehyde, metabolic transformations which would further complicate analysis are avoided, & pharmacological effects are kept to a minimum. Thus the amine which would be converted to PCA by amine oxidase (and thence to the other metabolites which PCA forms) might well itself undergo methylation, so that one could have:



(Methylation of the aldehyde is not shown in the diagram, but introduces a further possible complication).

An analysis of methylation in such circumstances would be formidable indeed : only a masochist would relish it.

Blockage of amine oxidation by M.A.O.I. might ease the analytical problem, but to administer a M.A.O.I. to volunteers and to schizophrenics would be unethical, because of the dangers of these drugs, which include worsening of the schizophrenic psychosis. The pharmacological action of the amine (whatever that may be) would be considerably potentiated by effective amine oxidase blockade : in short, such a project would be potentially dangerous, wholly unethical, and would introduce unnecessary analytical complications.

7. Chemical methods. A simple chemical estimation is available for the estimation of total paracetamol (free and conjugated) (q.v.). Although no method (except gas chromatography which was unavailable at the time of study) for detecting protocatechuic acid and its metabolites was available, no great difficulty was found in working a method out, although this method is admittedly somewhat cumbersome.

8. Suitability as judged by results in normal subjects. As far as the methacetin test is concerned, the histogram appearing in Fig. 17 p. 144 illustrates the point that there is space to the

left of the histogram where an abnormally low test result can be recognised as such (i.e. in the range 0 - 20%).

Similarly, with the PCA test, Figures 34 p 245and 35 p 246 illustrate the point that there is space to the right of the histogram for the "rapid methylators," should such exist. Furthermore, a relative increase of 4-O-methylation could appear to the left of the histograms given in Fig. 36 p 247.

9. See p 128.

10. Both tests are replicable (see p 162 and p 269).

11. Clearance of the drug. Rapid clearance of the drug offers certain advantages:- a) urine specimens will contain relatively high concentrations of metabolites; b) the test period can be kept short, an important consideration when supervising the mentally disturbed; c) a shorter interval will be possible between repeated tests on the same individual.

8. The choice of biological material to be analysed.

One question to which an answer had to be found at an early stage was whether to use blood or urine or both to follow the metabolism of the administered substances. Three questions had to be answered :-

1. Would psychiatrists having clinical responsibility for patients permit repeated venepuncture on acutely disturbed schizophrenics?

2. Would such venepuncture be ethical in view of the legal difficulty in defining 'valid consent' for this group of patients?
3. Would the patients themselves permit repeated blood analyses?
The author was advised at the outset that only urine should be examined because a) the answer to question 1 was generally likely to be "no" and b) the answer to question 3, "no" at least for some of the most disturbed patients (although by no means all). Question 2 bristles with difficulties. Those who committed themselves to an answer (the minority) all answered "no." Advice to restrict analysis to urine samples was accepted, albeit reluctantly. On its acceptance probably depended the subsequent satisfactory co-operation which was achieved, so that an adequate number of acutely disturbed patients were presented for testing. If blood samples had been insisted on, it seems doubtful whether some of these patients would have been presented by their psychiatrists as being suitable for testing and, indeed, whether some psychiatrists would have presented any patients for testing.

FINAL SUMMARY.

LITERATURE SURVEY.

1. The resemblance of schizophrenia to the clinical state induced by the methylated hallucinogenic amine mescaline led to the 'faulty transmethylation' hypothesis of Osmond and Smythies (1952), which has acted as a stimulus for much research.

2. Other methylated compounds such as dimethyltryptamine and L.S.D. share with mescaline the ability to induce hallucinations in a setting of clear consciousness; these three compounds show cross-tolerance.

3. Methyl donors (e.g. methionine) particularly when administered with a monoamine oxidase inhibitor (M.A.O.I.), can exacerbate schizophrenia. Symptoms of a toxic nature seem more likely to occur with very large doses of methyl donors plus M.A.O.I.. Cysteine (which is not a methyl donor) plus M.A.O.I. also appears to exacerbate schizophrenia, but cysteine may interfere with methionine metabolism by negative feed-back. Methyl donors and M.A.O.I. given together elevate levels of non-methylated amines more than does an M.A.O.I. given alone, indicating that methyl donor feeding does not result solely in increased transmethylation.

4. Dietary methyl donor restriction is reported to confer no benefit in schizophrenia, but the methodology employed is open to criticism.

5. An abnormality in the metabolism of C^{14} methyl-labelled methionine in schizophrenia has been reported. This abnormality would be consistent with a larger transmethylating pool in schizophrenics than in normal subjects.

6. A possible antimetabolite of methionine, methionine sulphoximine has been reported to cause a toxic psychosis in non-schizophrenic subjects, but to bring about improvement in chronic schizophrenics.

7. A report of benefit in schizophrenia from penicillamine, a substance structurally not unlike methionine has received little support. It has been suggested that penicillamine might act by blocking biochemical transformations involving transmethylation.

8. Claims have been made, but not widely accepted, for the efficacy of nicotinamide, a methyl acceptor, as a treatment for schizophrenia. The methodology employed to back up such claims can be regarded as less than satisfactory.

9. An association between a disorder of methionine metabolism, namely homocystinuria, and schizophrenia has been noted in several pedigrees. Such an association has not yet been established statistically.

10. The methylating enzyme catechol-O-methyl transferase has been administered parenterally to four moderately disturbed schizophrenic patients, with deterioration in their clinical state.

11. The claim made by Friedhoff and Van Winkle (1962) that schizophrenics excreted in the urine the methylated amine 3:4 dimethoxy phenylethylamine (D.M.P.E.) whereas normal subjects did not, has stimulated much research. Few now agree with Friedhoff's original assertion. Many investigators have not found D.M.P.E. in urine from normal or schizophrenic subjects. Some have found it in normals as well as in schizophrenics. Others have confirmed the presence on chromatograms of the pink spot that Friedhoff found and have suggested alternative identifications. Bourdillon et al. (1965) established an association between pink spot and schizophrenia for which no satisfactory explanation in terms of any form of artefact has yet been put forward.

12. D.M.P.E. given orally in dosage of up to 15 mgm/K has no psychotomimetic properties in man.

13. D.M.P.E. is metabolized chiefly by amine oxidase. 4-O-demethylation is a minor route of metabolism.

14. A number of studies have been made of D.M.P.E. synthesis from dopamine. The metabolism of dopamine is too complex for it to be an ideal substrate with which to study transmethylation. From none of these studies can one deduce that schizophrenics can synthesize D.M.P.E. from dopamine while controls cannot.

15. Reports of 5-hydroxy N:N dimethyltryptamine (Bufotenin) in urine of schizophrenics but not of controls are not convincing :

positive reports have frequently been associated with unsophisticated methodology.

16. Genetic studies tend to support a genetic basis for schizophrenia, but the mode of inheritance of schizophrenia has yet to be established.

17. Schizophrenia may prove to be an example of genetic polymorphism, but this is conjectural. The nature of any biological advantage conferred on non-schizophrenic carriers of genes for schizophrenia has not been established.

18. The concept of schizophrenia as an example of genetic polymorphism would be consistent with a primary biochemical disturbance extending beyond the brain itself. Knowledge concerning the profound psychic disturbances which can occur in disorders classified as inborn errors of metabolism is consistent with such a concept of schizophrenia (e.g. acute intermittent porphyria).

EXPERIMENTAL SECTION 1.

19. The hypothesis is put forward that, in some or all schizophrenics, a deficiency in demethylation allows methylated compounds to accumulate.

20. O-demethylating enzymes appear to occur only in liver microsomes.

21. Psychopharmacological studies indicate that the possession of a 4-methoxy group is necessary but not sufficient for a phenylethylamine to possess a mescaline-like effect. Demethylation

by a liver microsomal enzyme occurs mainly in the 4 position. This can be regarded psychopharmacologically as a detoxicating process.

22. Human liver demethylating enzyme studies are presented.

23. Demethylation leads to the formation of formaldehyde for which a convenient method of estimation exists. This method is validated for human liver.

24. Human liver is shown to require T.P.N. and nicotinamide as cofactors; to demethylate D.M.P.E. in the 4 position; and to demethylate methacetin.

25. D.M.P.E. and methacetin appear to be demethylated by the same enzyme; a different enzyme demethylates codeine.

26. Difficulties were experienced with human liver using the methods described. Contamination with formaldehyde, opalescence due to fat and sluggish activity are commented on. Liver from schizophrenics proved elusive for a number of reasons.

EXPERIMENTAL SECTION 2.

27. The use of pharmacogenetics (which can be defined as the study of genetically determined variations in animal species which are revealed by the effects of drugs) suggested itself as a way of circumventing the difficulties mentioned above.

28. Methacetin was chosen as a suitable drug for a pharmacogenetic study of the demethylation hypothesis since 4-O-demethylation is

the major route of degradation of this compound. The O-demethylated product of methacetin is a familiar drug : paracetamol.

29. Proliminary experiments established the validity of the various laboratory procedures employed in the estimation of methacetin. Pharmacokinetic studies indicated a drawback : that subsequent metabolic steps (i.e. the formation and excretion of paracetamol conjugates) are rate-limiting.

30. The procedure for the administration of methacetin is described; this is the "Methacetin test."

31. This test was applied to 53 normal subjects, 24 acute schizophrenics and to two groups, each of 31 mental hospital patients, one group comprising schizophrenics, the other comprising non-schizophrenics.

32. With one exception, such differences as were found were attributable to factors other than a diagnosis of schizophrenia.

33. The effects of prior consumption of phenothiazines on the methacetin test results appears to be unimportant.

34. The methacetin test yielded abnormal results in patients ill with non-psychiatric disorders including malabsorption, liver disease and renal disease.

35. In mental hospital patients the methacetin test showed satisfactory replicability.

36. Difficulties encountered during methacetin testing in

psychotic patients were infrequent; these are described.

37. Findings in a single schizophrenic showing abnormal methacetin test results are discussed; the possibility of error in urine collection cannot be absolutely excluded.

EXPERIMENTAL SECTION 3.

38. Transmethylation was studied by the administration of 3:4 dihydroxybenzoic acid (protocatechuic acid, PCA). In the dosage given, PCA was expected to produce no subjective effects; subsequent findings accorded with this expectation.

39. The metabolism of this compound involves methylation in either the 3-O or the 4-O position, producing vanillic acid (VA) and isovanillic acid (IVA) respectively. Both VA and IVA are conjugated with glycine, yielding vanillylglycine (VAG) and isovanillylglycine (IVAG) respectively. After oral PCA administration PCA, VAG, VA, IVAG and IVA all appear in the urine.

40. These metabolites were identified by a variety of chromatographic methods. Amounts of IVA (i.e. of unconjugated IVA) were too small to be measured conveniently.

41. PCA glucuronide is considered to be, at most, a minor metabolite of PCA. Evidence of 4-dehydroxylation of PCA was not found.

42. Methods for estimating PCA, free and total VA and the ratio VA/IVA are described. Interference by phenolic acids other than

PCA and its metabolites was measured and found to be small.

43. A gas chromatographic method of estimating 3:4 dimethoxy-benzoic acid (veratric acid, VRA) in extracts from urine containing PCA and VA in excess is described. In a limited study VRA was present in urine extracts obtained after PCA administration in amounts below the limits of detection by the method used (usually $\frac{\text{VA (free and conjugated)}}{\text{VRA(" " ")}}$ was >100).

44. A standard method of administering protocatechuic acid (and subsequently of collecting urine) is described (the PCA test). The test was administered to 42 normal males, 13 normal females, 11 male schizophrenics and 10 female schizophrenics. All the schizophrenics were in the "shifting phase" of their illness. On each urine sample measurements were made of volume, pH, creatinine and PCA. The total amounts of vanillic acid and isovanillic acid (total = free and conjugated) were also estimated in all cases. On 30 urines from control subjects and all urines from schizophrenics a measurement of unconjugated VA was made. This measurement gives only an approximate result, making no allowance for the presence of small amounts of unconjugated IVA.

45. Statistical evaluation of results showed that:-

a) there were no significant differences between schizophrenics and normal subjects in $\frac{\text{VA (total)}}{\text{PCA}}$ % or $\frac{\text{IVA (total)}}{\text{PCA}}$ %.

b) $\frac{VA(total)}{PCA}$% was dependent on body weight in females, both in schizophrenics and normals. The data was to some extent consistent with the hypothesis that VA penetrates into body fat.

c) $\frac{VA(total)}{PCA}$ % and $\frac{IVA(total)}{PCA}$ ratios showed highly significant correlation ($p<0.001$) in all groups studied.

d) $\frac{VA\ (total)}{PCA}$ % was related directly to $\frac{IVA\ (total)}{VA(total)}$. It is suggested that both may be related to the availability of methyl groups.

e) Unconjugated vanillic acid excretion varied directly with urinary pH.

46. The total recovery of metabolites showed excellent agreement between the four groups studied. This indicates a satisfactory level of patient co-operation.

47. Prior consumption of phenothiazines appeared to have no effect on PCA test results.

48. Satisfactory replicability for the ratios $\frac{VA\ (total)}{PCA}$ % and $\frac{IVA\ (total)}{PCA}$% was established in 12 normal subjects.

49. There was more scatter in the values obtained for three hour urinary creatinine in schizophrenics than in normal subjects. This may related in part to the clinical state, agitated and frightened patients having high values, apathetic, energic and inert patients, low values.

50. The results of PCA testing are compared with those of other

studies. The present findings have some implications for future research:-

a) the suggestion that the methylated compound vanillic acid penetrates body fat stimulates one to consider whether other compounds, including lipid soluble amines, may do likewise. Such a hypothesis, could, if related to tryptamine, readily be investigated. Other corrolaries that seem worth investigation are also discussed.

b) The correlation between $\frac{VA(total)}{PCA}$, and $\frac{IVA\ (total)}{VA\ (total)}$ should be investigated further using in vitro methods; in the present study the relationship is put forward tentatively, being of a low order of significance statistically.

c) Further investigations into the relationship between urinary creatinine and the clinical symptoms of schizophrenia would seem to be indicated.

DISCUSSION OF THE PHARMACOGENETIC APPROACH.

51. An assessment is made of the suitability of methacetin and protocatechuic acid for the purposes for which they were used. Both compounds have advantages and disadvantages, as has the pharmacogenetic method itself.

APPENDIX 1 : METHACETIN TEST RESULTS

TABLE XXIV Results of Methacetin Test in Control Subjects.

N.B. Percentage demethylated = Amount of demethylated metabolites in the 4 hour urine collection (expressed as mgms. methacetin) x 100 divided by dose in mgms.

Male Subjects.

No.	Age yrs.	Weight lbs.	Dose mgms.	Urine Vol. mls.	Percentage demethylated
1	24	178	612	510	48.3
2	21	167	577	780	24.0
3	19	134	490	400	32.3
4	19	134	490	450	43.6
5	23	128	472	960	27.4
6	24	161	560	1080	20.4
7	22	143	525	760	46.3
8	20	217	700	995	21.3
9	19	154	542	700	30.5
10	18	163	577	620	40.5
11	20	146	525	620	28.7
12	20	136	507	950	34.0
13	20	160	560	880	35.5
14	23	139	507	805	41.1
15	21	139	507	755	44.1
16	20	171	595	840	39.3
17	19	177	595	660	39.1
18	20	140	507	395	39.6
19	20	129	490	880	44.9
20	29	174	595	695	33.2
21	22	160	560	695	38.5
22	20	138	507	940	31.1
23	19	161	560	1310	44.0
24	19	163	577	1300	34.4
25	20	146	525	850	42.9

Male Subjects cont/d.

No.	Age yrs	Weight lbs.	Dose mgms	Urine vol. mls.	Percentage demethylated.
26	24	174	595	410	37.7
27	18	140	507	540	48.7
28	17	137	507	610	40.4
29	20	133	490	490	45.1
30	27	188	630	570	55.9
31	43	182	612	490	50.8
32	20	134	490	1240	34.8
33	26	160	560	520	46.1
34	18	147	525	870	40.9
35	20	168	577	630	41.6
36	20	144	525	830	34.7
37	21	169	577	910	48.7
38	20	228	717	720	40.7
39	20	138	507	1310	46.1
40	22	154	542	890	43.9
41	21	140	507	820	36.5
42	20	194	630	670	41.5
43	20	152	542	470	41.2
44	26	166	577	970	25.9
45	20	148	525	900	46.4
46	19	154	542	300	44.6
47	22	142	507	790	37.4
Female Subjects.					
1	22	113	437	1200	27.7
2	22	140	507	920	50.2
3	22	137	507	1160	32.1
4	23	120	455	470	56.4
5	22	172	595	560	45.6
6	29	137	507	480	40.5

Table 25. RESULTS for ACUTE SCHIZOPHRENICS. (Appendix 4 gives clinical details).

No.	Initials	Sex	Age Years	Weight Lb.	Dose mgms	Urine Volume mls	Percentage demethylated	Drugs prior to testing. See Note 1. Time. Drug.	Pink Spot Status. See Note 2.
1.	J.A.	M	19	140	507	895	31.0	2 days T,S	(+)
2.	P.D.	F	19	112	437	1260	48.4	2 days T	-
3.	D.B.	M	28	220	700	455	36.6	10days C	-
4.	A.C.	M	20	142	507	350	32.7	10days S	(+)
5.	G.P.	M	26	136	507	570	26.6	10days C	-
6.	J.W.	M	23	180	612	180	33.3	10days T	-
7.	P.P.	F	37	149	542	765	40.4	3 mths S	-
8.	W.L.	M	18	136	507	250	23.2	No drugs	-
9.	K.A.	F	16	136	507	680	24.4	Doubtful S	-
10.	A.G.	M	43	164	577	1160	38.0	Doubtful C	-
11.	R.F.	M	33	163	577	620	26.8	14mths S,A(4)	-
12.	M.B.	M	37	128	472	650	42.5	14days S	-
13.	A.M.	M	24	132	490	265	27.4	No drugs	-
14.	B.K.	M	28	149	542	360	39.3	3 mths S	+
15.	E.R.	M	29	138	507	290	38.8	3 mths S	-
16.	V.W.	M	27	128	472	125	44.2	8weeks C	+
17.	W.H.	M	37	151	542	460	31.7	4 mths T,A(2)	-
18.	D.M.	M	36	137	507	375	40.2	4 mths F	-
19.	S.B.	M	40	145	525	500	52.0	4weeks S	-

cotd.

Table 25 ctd.

No.	Initials	Sex	Age Years	Weight Lb.	Dose mgms	Urine Volume mls	Percentage demethylated	Drugs prior to testing. See Note 1. Time. Drug.	Pink Spot Status. See Note 2.
20.	C.F.	M	38	162	560	120	55.5	3 weeks S	-
21.	D.D.	M	36	132	490	185	36.3	10 mths S,A(4)	-
22.	S.W.	M	18	112	437	215	47.4	No drugs	+
23.	R.W.	M	31	214	682	285	29.3	Doubtful	-

NOTE 1. Time :- Probable drug-free period prior to testing.

Drugs :- The key is as follows-

T = THIORIDAZINE (Melleril)

S = TRIFLUOPERAZINE (Stelazine)

C = CHLORPROMAZINE (Largactil)

F = FLUPHENAZINE (Moditen)

A = Sodium Amytal.

Sodium amytal was given the night before, or two nights, before the test, in a <u>total</u> dose (in hundreds of mgms) after the figure A.

NOTE 2. ✱ = Pink Spot found at the time of the methacetin test.

(+) = Pink Spot found at some other time.

- = Pink Spot never found.

TABLE XXVa Results for Chronic Schizophrenics.

Initials	Sex	Age	Weight lbs	Dose mgms	Urine vol. mls.	Percentage demethylated	Length of time off drugs. (see Note 1)
R. McD.	M	64	135	490	600	29.0	N.R.
T.M.W.	M	59	169	577	985	32.7	N.R.
J.T.	M	60	130	490	940	29.2	N.R.
J.H.	M	59	120	455	990	43.0	3½ yrs.
F.W.	M	53	126	472	445	43.7	4 yrs.
T.H.	M	66	128	472	297	40.1	N.R.
C.D.	M	60	123	472	540	39.3	N.R.
F.L.	M	67	127	472	740	34.4	4 yrs.
M.H.	F	48	94	385	363	25.5	N.R.
H.G.	F	55	147	525	490	33.3	1 yr.
E.H.	F	67	103	420	136	26.0	N.R.
M.G.	F	59	156	560	698	41.6	N.R.
A.F.	F	54	112	437	170	13.2	1 yr.
L.C.	F	69	133	490	465	21.3	N.R.
F.F.	M	59	126	472	215	29.0	2 yrs.
C.F.	M	54	171	595	915	30.7	3 yrs.
D.K.	M	59	147	525	980	30.1	2 yrs.
G.S.	M	52	136	507	930	30.2	1½ yrs.
J.C.C.	M	66	105	420	580	30.0	N.R.
J.H.	M	47	172	595	362	32.2	1 yr.
C.M.	M	55	116	455	201	29.6	2 yrs.
M.D.	F	45	126	472	370	20.2	2 yrs.
J.M.	F	57	102	402	1012	29.7	N.R.
J.J.	M	57	125	472	612	41.5	N.R.

TABLE XXVa Cont/d.

Initials	Sex	Age	Weight lbs.	Dose mgms	Urine Vol. mls.	Percentage demethylated	Length of time off drugs. (see note 1).
E.G.	M	52	188	630	702	41.8	5 yrs.
J.G.	M	58	104	420	433	32.2	6 yrs.
A.B.	M	60	107	420	484	37.6	5 yrs.
J.C.	M	45	154	542	789	17.1	N.R.
A.R.	F	62	126	472	570	27.1	2 yrs.
M.W.	F	57	134	490	777	36.6	2 yrs.
M.J.	F	62	154	542	233	29.2	N.R.

Note 1. N.R. = No record of a course of psychotropic drugs having been given. This applies to Table XXVb also.

Note 2. In none of the patients was there any record of barbiturate or phenothiazine therapy in the twelve months prior to testing.

TABLE XXVb Results for Non-schizophrenic Mental Hospital Patients.

Initials & Diagnosis*	Sex	Age yrs.	Weight lbs.	Dose mgms	Urine Vol mls.	Percentage demethylated	Length of time off drugs. (see Note 1).
B.B. 6	M	42	142	507	692	40.5	N.R.
R.F. 6	M	69	131	490	452	19.1	N.R.
F.M. 2	M	58	126	472	303	46.7	9 yrs.
F.S. 3	M	72	168	577	413	22.7	N.R.
R.A. 6	M	65	144	525	710	25.4	N.R.
J.C. 8	M	58	134	490	373	23.8	N.R.
R.L. 5	M	52	130	490	457	41.6	4 yrs.
R.M. 6	M	68	134	490	484	18.5	N.R.
H.D. 6	M	43	124	472	904	35.9	N.R.
C.R. 6	M	64	114	437	895	34.7	18 yrs.
C.C. 6	M	65	98	402	320	25.2	2½ yrs.
J.S. 6	M	46	155	542	653	36.8	N.R.
J.J. 3	M	71	117	455	227	23.6	9 months.
L.J. 3	M	69	125	472	296	22.1	4 yrs.
M.W. 3	M	50	145	525	247	28.8	7 months.
R.G. 4	M	64	149	542	725	28.5	1½ yrs.
T.U. 2	M	51	170	577	376	20.3	2 yrs.
J.U. 6	M	54	138	507	734	29.2	N.R.
W.G. 3	M	56	142	507	352	47.0	N.R.
G.A. 6	M	40	128	472	1163	21.4	N.R.
M.B. 6	F	42	98	402	560	30.1	N.R.
J.B. 6	F	68	96	385	572	30.1	2 yrs.
G.G. 6	F	35	98	402	1003	39.1	4 yrs.
M.A. 6	F	49	138	507	975	36.7	N.R.
R.H. 9	F	63	107	420	864	20.4	1 yr.
E.M. 6	F	61	197	647	564	26.7	14 yrs.

*see overleaf.

TABLE XXVb cont/d.

Initials & Diagnosis*	Sex	Age yrs	Weight lbs.	Dose mgms	Urine vol. mls	Percentage demethylated	Length of time off drugs. (see note 1).
M.W. 7	F	65	116	455	437	19.3	2 yrs.
M.B. 6	F	74	140	507	387	30.5	N.R.
E.B. 6	F	64	135	490	540	12.8	2 yrs.
E.R. 9	F	49	220	700	493	27.1	2 yrs.
M.C. 6	F	66	140	507	280	24.1	2½ yrs.

*The number after the initals gives the diagnosis, according to the following key:-

2. Epileptic dementia
3. G.P.I.
4. "Hysterical psychopathy."
5. Korsakov psychosis.
6. Mental subnormality.
7. Meningo-vascular syphilis.
8. Post-encephalitic dementia.
9. Recurrent depression.

Note 1: In none of the patients was there any record of barbiturate or phenothiazine therapy in the seven months prior to testing.

APPENDIX 2 : PCA TEST RESULTS

TABLE 26. PCA Test : Results for Control Subjects.

Male Subjects.

No.	Age	Weight lbs.	Dose mgms	Urine vol. mls.	Urinary pH	Creatinine mgms.
1	19	124	900	690	6.8	186
2	19	145	1050	508	5.6	227
3	23	153	1100	142	5.2	293
4	33	140	1000	589	6.4	287
5	18	159	1150	345	6.5	231
6	19	169	1200	238	5.8	244
7	23	154	1100	456	5.2	267
8	21	206	1500	278	6.6	236
9	20	142	1000	68	5.9	187
10	21	180	1300	525	5.7	321
11	21	163	1200	618	6.6	272
12	19	179	1300	443	5.3	152
13	19	161	1150	620	7.1	156
14	22	139	1000	427	7.0	180
15	22	156	1100	472	6.3	194
16	32	171	1250	338	7.0	204
17	21	178	1300	293	7.7	240
18	21	143	1000	438	6.3	271
19	19	153	1100	96	5.4	184
20	19	147	1050	707	6.4	191
21	18	157	1150	642	6.2	232
22	20	149	1050	358	6.7	127
23	18	147	1050	346	5.9	240
24	20	162	1150	238	6.5	162
25	20	135	950	95	5.7	195
26	28	149	1050	455	5.9	149
27	23.	227	1650	268	6.1	215
28	18	155	1100	174	5.9	230

TABLE 26 cont/d.

Male Subjects

No.	PCA mgms.	VA(total)% / PCA	IVA(total)% / PCA	VA / IVA	$Log_{10}\frac{VAG}{VA}$
1	516	49.0	3.18	15.4	-
2	502	54.4	4.06	13.4	-
3	580	42.8	3.36	12.7	1.279
4	483	59.2	4.45	13.3	0.556
5	695	37.3	2.85	13.1	-
6	644	39.4	2.42	16.3	0.960
7	450	73.8	6.16	12.0	1.382
8	555	63.1	5.64	11.2	-
9	408	49.8	3.26	15.3	-
10	609	50.6	4.06	12.5	0.985
11	616	49.5	4.81	10.3	0.685
12	645	45.9	4.06	11.3	-
13	393	85.5	7.25	11.8	-
14	315	88.6	5.52	16.0	1.201
15	484	66.3	6.45	10.3	-
16	478	67.4	6.82	11.6	-
17	676	51.9	3.88	13.4	-
18	566	44.2	3.23	13.7	0.902
19	451	63.2	5.25	12.0	1.009
20	580	51.0	3.90	13.1	0.614
21	529	65.8	5.10	12.9	1.057
22	219	85.8	6.85	12.5	1.045
23	463	70.2	4.17	16.9	-
24	608	45.7	4.16	11.0	0.790
25	466	49.1	3.76	13.1	0.743
26	342	55.8	4.82	11.6	-
27	402	68.9	6.22	11.1	-
28	551	38.3	2.12	18.0	0.762

TABLE 26. cont/d.

No.	Age	Weight lbs.	Dose mgms	Urine vol. mls.	Urinary pH	Creatinine mgms.
			Male Subjects			
29	19	139	1000	169	6.3	195
30	19	147	1050	407	5.9	163.
31	18	156	1100	90	5.4	206
32	19	144	1050	373	5.6	200
33	24	149	1050	598	5.7	193
34	27	130	900	403	6.0	293
35	19	131	950	388	5.4	193
36	19	117	800	228	7.0	196
37	20	165	1200	430	6.8	231
38	20	147	1050	210	5.7	211
39	20	156	1100	485	6.6	207
40	20	177	1300	444	5.3	263
41	23	146	1050	1530	5.9	201
42	19	159	1150	773	5.4	213
			Female Subjects			
1	19	129	900	870	7.2	202
2	20	133	950	482	7.0	293
3	20	147	1050	520	5.2	153
4	24	157	1150	390	5.6	194
5	29	150	1050	265	5.4	213
6	36	126	900	486	5.9	135
7	22	112	800	308	6.2	118
8	26	152	1100	105	5.2	156
9	32	118	850	701	6.4	150
10	23	129	900	101	5.0	139
11	19	136	950	560	6.5	183
12	20	142	1000	362	5.8	159
13	19	126	900	659	6.4	253

TABLE 26 cont/d.

No.	PCA mgms	VA(total)% / PCA	IVA(total)% / PCA	VA / IVA	$\log_{10}\frac{VAG}{VA}$
			Male Subjects		
29	511	43. 4	3.17	13.7	-
30	526	49.2	3.38	14.6	0.990
31	502	53.4	3.73	14.3	1.045
32	343	82.2	6.27	13.1	-
33	608	51.2	4.10	12.5	0.876
34	442	67.0	7.26	9.2	0.892
35	479	55.7	4.28	13.1	1.083
36	460	50.7	4.74	10.7	0.698
37	601	48.6	4.23	11.5	-
38	517	51.1	5.32	9.6	-
39	498	66.5	7.15	9.3	0.628
40	590	64.6	6.10	10.6	1.060
41	528	67.4	5.08	13.3	-
42	595	51.3	4.67	11.0	0.816
			Female Subjects		
1	518	48.1	3.22	14.9	-
2	496	46.2	3.67	12.6	-
3	405	39.8	3.14	12.7	1.061
4	615	39.0	2.05	19.0	-
5	628	41.2	3.77	10.9	-
6	430	58.6	5.05	11.6	-
7	418	56.7	4.74	12.0	0.834
8	600	39.2	3.22	12.2	1.114
9	471	54.8	3.35	16.3	-
10	399	59.6	5.91	10.1	1.204
11	587	43.4	3.95	11.0	0.653
12	462	60.4	5.54	10.9	0.658
13	380	80.0	6.66	12.0	-

TABLE 27 : PCA Test : Results for Schizophrenics.

Code No.	Initials	Sex	Age	Wt. lbs.	Dose moms.	Urine vol. mls.	Urinary pH
31*	E.G.	M	30	152	1100	700	6.7
32	A.T.	M	41	161	1150	135	5.1
36**	S.W.	M	21	111	750	403	6.9
39	C.P.	M	25	128	900	315	6.5
40	T.H.	M	22	130	900	220	6.5
41	W.A.	M	19	110	750	225	7.0
42	M.G.	M	30	92	650	505	6.9
43	C.J.	M	17	119	850	135	5.9
44	P.G.	M	53	132	950	930	7.2
45	D.G.	M	24	180	1300	1030	6.8
46	H.T.	M	34	181	1300	730	6.8
33	E.R.	F	39	104	750	855	6.2
34	D.D.	F	30	80	550	59	5.3
35	W.C.	F	32	110	750	610	6.7
37	J.W.	F	28	104	750	125	6.3
38	B.W.	F	42	115	800	680	7.1
47	B.F.	F	28	153	1100	81	5.5
48	E.H.	F	64	143	1000	158	6.9
49	B.H.	F	21	125	900	920	7.8
50	D.P.	F	29	99	700	655	6.2
51	D.K.	F	22	128	900	688	7.0

* = Pink Spot positive at time of testing.

** = Pink Spot positive at some time (but not at time of test).

TABLE 27 cont/d.

Code No.	Creatinine mgm.	PCA mgms	VA(total) / PCA %	IVA(total) / PCA %	VA / IVA	$Log_{10}\frac{VAG}{VA}$
31	55	551	48.1	3.54	13.6	1.396
32	129	274	72.3	6.46	11.2	0.997
36	154	298	51.7	3.32	15.5	0.538
39	221	577	40.4	3.15	12.8	1.356
40	294	437	62.9	7.07	8.9	0.930
41	225	471	37.6	2.38	15.8	0.771
42	234	267	72.3	7.45	9.7	0.900
43	219	375	44.0	3.09	14.2	0.933
44	166	423	50.4	4.66	10.8	0.780
45	704	696	51.7	4.11	12.6	0.688
46	228	595	66.3	6.15	10.8	0.655
33	120	457	45.1	2.84	15.8	0.961
34	91	170	94.7	8.94	10.6	1.258
35	122	493	40.0	3.89	10.3	0.799
37	109	238	79.3	7.43	10.7	0.634
38	136	515	38.4	3.77	10.2	0.789
47	256	582	34.0	3.33	10.2	1.810
48	138	404	37.1	2.72	13.7	0.970
49	129	491	66.6	6.80	9.8	0.736
50	79	262	61.1	5.61	10.9	1.072
51	339	548	58.0	4.03	14.4	1.140

APPENDIX 3 Protocatechuic Acid Test : Instructions for Making Up the Test Solution.

1. Subtract 2 lbs. for pyjamas (both sexes) and for indoor clothes (excluding shoes) subtract 3 lbs. for females and 5 lbs. for males from the uncorrected weight of the subject.
2. Check the constitution of the solution against the weight of the subject.
3. The PCA should be weighed out accurate to the nearest mgm. in a beaker large enough to hold the final volume.
4. Add a volume of tap water from a measuring cylinder equal to the total volume MINUS the volume of NaOH.
5. Stir and warm gently until the PCA has dissolved.
6. Add the glucose. Weigh this out to the nearest 0.1 gram, stir to dissolve.
7. Finally add the appropriate amount of 1 N NaOH. Stir thoroughly and check that the final solution is neutral by applying the stirring rod to pH paper.
8. Pour the solution into a clean medicine bottle, label with the subject's name and store in a refrigerator until required.

BODY WEIGHT (lbs).	PCA (mgms)	N NaOH (mls)	GLUCOSE (G)	TOTAL VOL. (mls)
76-82	550	3.3	4.1	110
83-89	600	3.6	4.5	120
90-96	650	3.9	4.9	130
97-103	700	4.2	5.2	140
104-111	750	4.5	5.6	150
112-117	800	4.8	6.0	160
118-123	850	5.1	6.3	170
124-130	900	5.4	6.7	180
131-136	950	5.7	7.1	190
137-143	1000	6.0	7.5	200
144-150	1050	6.3	7.8	210
151-156	1100	6.6	8.2	220
157-162	1150	6.9	8.6	230
163-169	1200	7.2	9.0	240
170-175	1250	7.5	9.3	250
176-182	1300	7.8	9.7	260
183-188	1350	8.1	10.1	270
189-195	1400	8.4	10.4	280
196-203	1450	8.7	10.8	290
204-210	1500	9.0	11.2	300
211-217	1550	9.3	11.6	310

Appendix 4 Brief details of the clinical features of the acute schizophrenics methacetin tested and of all schizophrenics PCA tested.

At the time the tests were carried out, the author was unversed in psychiatric terminology and unfamiliar with psychiatric assessment. He therefore transcribed from the patient's case sheet those words or phrases which seemed to indicate the areas of chief psychopathology. The object of this appendix is to outline each case so as to indicate the clinical features on which the diagnosis of schizophrenia largely depended. Unless otherwise stated, the symptoms were those present at the time of the test; it will be evident that some very disturbed patients were persuaded to submit to the test procedures.

Age is the age of the patient at the time of testing.

Onset →test is the length of time between the first symptoms and the test. F.H. is family history; F.H.-ve indicates no family history of schizophrenia. Off drugs →test is the probable length of time prior to testing that phenothiazines were discontinued (see also Tables 25 and 27).

Key to drugs : C = chlorpromazine

F = fluphenazine

S = trifluoperazine (=Stelazine)

T = thioridazine

1. J.A. Male. 19 yrs. Onset → test : 3 months. F.H.-ve. Auditory hallucinations; ideas of reference; thought blocking and over-inclusive thinking. Off drugs (T and S) → test : 48 hours. Response to phenothiazines : good. Discharged 3/52 after testing much improved. Not readmitted in subsequent 18/12.

2. P.D. Female. 19 yrs. Onset → test : 12 months. F.H. -ve. Tense, agitated, auditorily hallucinated; incongruous affect; inappropriate smiling. Off drugs (T) → test : 48 hours. Response to phenothiazines fair. Readmitted 15 months after testing with auditory hallucinations, delusions. Manneristic, affect flat. Subsequently transferred to a chronic ward (two years after test).

3. D.B. Male. 28 yrs. Onset → test : 10 years. F.H. -ve. Hypochondriacal delusions, auditory hallucinations, gross thought disorder. Many admissions and discharges over the years. Tested in remission. Off drugs (C) → test : 10 days. Several subsequent readmissions.

4. A.C. Male. 20 yrs. Onset → test : 4 years. F.H. -ve. At onset, perplexed, doluded, manneristic. A year later apathetic with flattened affect. At time of testing aggressive with delusions of persecution. Diagnosed as paranoid schizophrenia. Off drugs (S) → test : 10 days. Ten months after testing while on the M.A.O.I. Isocarboxazid experienced auditory hallucinations

and simultaneously became pink spot positive (previously pink spot negative). Several subsequent readmissions, terminating in transfer to a chronic ward. Responded poorly to phenothiazines.

5. G.P. Male. 26 years. Onset → test : 18 months. F.H. +ve, (mother firmly diagnosed as schizophrenic). Acute onset with auditory hallucinations, blasphemous phonemes; delusions; depressed mood. Off drugs (C) → test : 10 days. Tested while in partial remission. Subsequently lost sight of.

6. J.W. Male. 23 yrs. Onset → test : 5 years. F.H. +ve, (mother, brother, sister and paternal uncle firmly diagnosed as schizophrenic). Original features : thought disorder, auditory hallucinations, childish and slovenly behaviour. At test : withdrawn, inaudible, restless, apparently hallucinated, untidy, silly. Off drugs (T) → test : 10 days.
Fair response to phenothiazines. Several subsequent readmissions culminating in transfer to chronic ward 10 months after testing.

7. P.P. Female. 37 yrs. Onsdt → test : 10 - 12 yrs. F.H. -ve. Aggressive, strange in manner, uncooperative, Previous auditory hallucinations. Thought disorder present. Off drugs - (S) → test : 3 months. Good response to phenothiazines. Discharged 3 months after testing and not readmitted in

subsequent 2 yrs. to the hospital in which she was tested.

8. U.L. Male. 18 yrs. Onset → test : 1 month. F.H. -ve. Fatuous, bizarre behaviour; delusions of persecution; manneristic and grimacing when interviewed. No drugs prior to testing. Subsequently responded well to chlorpromazine; discharged after 5 months and not readmitted (to the hospital in which he was tested) in subsequent 2 years.

9. K.A. Female. 16 yrs. Onset → test : 12 months. F.H. -ve. Onset with depressed mood. Readmitted at time of test tense, restless, muttering to hallucinatory voices, sullen, negativistic. Off drugs (S) → test : probably several months. Fair response to phenothiazines but several subsequent readmissions with marked auditory hallucinations.

10. A.G. Male. 43 yrs. Onset → test : 12 yrs. F.H. -ve. Several admissions prior to testing. At time of test delusions of persecution; probably hallucinated (auditorily); thought blocking present. Off drugs (C) → test : doubtful. Several subsequent readmissions when frankly hallucinated.

11. R.F. Male. 33 yrs. Onset test → 16 yrs. F.H. -ve, for schizophrenia. One brother mentally subnormal.
Onset with abusive phonemes. 8 yrs. later : readmitted, manneristic, thought disordered, blunting of affect. Low I.Q. (W.A.I.S. 76). At time of testing : withdrawn, manneristic.

Off drugs (S) → test 14/12 probably. Poor response to phenothiazines. Subsequently readmitted and transferred to a more chronic ward.

12. M.B. Male. 37 yrs. Onset → test : 5 yrs. F.H. of psycho-neurosis but not of schizophrenia. Nervous, agitated, delusions of persecution, thought broadcasting, blunting of affect. Off drugs → test : 2 weeks. Fair response to phenothiazines. Not subsequently readmitted to hospital where tested (2 yr. follow-up).

13. A.M. Male. 24 yrs. Onset → test "a few months." F.H. -ve. Failure of concentration, declining work record, odd, slow, thought disordered. Some obsessional thoughts, e.g. "bugger God." No record of drugs prior to test. Fair response to phenothiazines. A year later : readmitted with "acute exacerbation of chronic schizophrenia" but notes very scanty.

14. B.K. Male. 28 yrs. Onset → test : 2 yrs. F.H. Adopted child. At onset : delusions of persecution. At test : perplexed, auditory hallucinations, deluded, thought withdrawal. Off drugs (S) → test : 3 months probably. A seaman, the patient subsequently sent from various ports letters suggestive of continuing schizophrenia; not subsequently readmitted.

15. E.R. Male. 29 yrs. Onset → test : 4 yrs. F.H. -ve. At onset : hallucinated, garrulous, knight's-move thinking. A year later :

phonemes discussing him in the third person; aggressive towards these phonemes. At test : delusions of persecution; thought disorder. Hallucinations denied. Passivity experiences. Off drugs (S) → test : 3 months probably. Good response to phenothiazines. Subsequently lost sight of.

16. V.W. Male. 27 yrs. Onset → test : 6 months. F.H. not recorded. Hypochondriacal delusions. Auditory hallucinations: "a woman's voice." Laughing, giggling. Off drugs (C) → test : 8 weeks. Poor response to phenothiazines. Slight improvement after E.C.T. Subsequently returned to Eire.

17. W.H. Male. 37 yrs. Onset → test : 6 yrs. F.H. -ve. At test : sullen, uncooperative, aggressive when disturbed; affect flattened; subsequent auditory hallucinations and persecutory delusions. Off drugs (T) → test : 4 months probably. Phenothiazines led to improvement and discharge after four months. Not readmitted to the same hospital in subsequent 18 months.

18. D.M. Male. 36 yrs. Onset → test : 13 years. F.H. -ve. Four years before test : mute, inaccessible, flexibilitas cerea. At test : quiet, withdrawn, auditorily hallucinated with abusive phonemes; negativistic. Off drugs (F) → test : 4 months probably. Good response to phenothiazines and E.C.T. Not readmitted to same hospital in subsequent 18 months.

19. S.B. Male. 40 yrs. Onset ⟩ test : 16 yrs. F.H. +ve, sister firmly diagnosed as schizophrenic. Admitted 4 yrs. after onset with delusions of persecution; aggressive, confused, thought blocking, auditory hallucinations. At time of test : euphoric, aggressive, delusions with delusional perception. Off drugs (S) ⟩ test : probably 3 - 4 weeks. Good response to phenothiazines. Discharged well a month after testing. Remained well on out-patient phenothiazines (2 yr. follow-up).

20. C. F. Male. 38 yrs. Onset ⟩ test : 4 yrs. F.H. -ve. One yr. after onset : deluded; obsessed with religious ideas; thought alienation. At test : deluded, worried, anxious. Exacerbation due to his stopping drugs, (Trifluoperazine/Artane) 3 weeks before admission. Good response to phenothiazines and satisfactory out-patient progress (18 months follow-up).

21. D.D. Male. 36 yrs. Onset ⟩ test : approximately 9 yrs. F.H. not obtained. 2 yrs. after onset diagnosed as paranoid schizophrenia. At test : guarded, withdrawn, apathetic, delusions of persecution, auditorily hallucinated. Off drugs (S) ⟩ test : 10 months. Fair response to phenothiazines. Subsequently lost sight of.

22/36. S.W. Male. 18 yrs, old when methacetin tested, 21 when PCA tested. F.H. unavailable, patient adopted.

Onset → methacetin test : 3 months.

Onset → PCA test : 2 yrs. 7 months.

At methacetin test : grimacing, grinning, delusions of grandeur, thought blocking. Little spontaneous speech. Hallucinations doubtful. No drugs prior to methacetin test. At PCA test : withdrawn, grossly thought disordered, apathetic, manneristic posture. Off drugs (S) → PCA test : 5 weeks. Again very poor response to phenothiazines. A grossly disorganised individual, who is now in ward for chronic psychotic patients.

23. R. W. Male. 31 yrs. Onset → test : 18 months. F. H. report (unconfirmed) of schizophrenia in a first cousin once removed. Auditory hallucinations, delusions. At test : grossly deluded, hallucinated, passivity experiences, thought disordered. Off drugs - test : doubtful, probably very irregular trifluoperazine prior to testing. Response to phenothiazines : good. Discharged 2 months after testing. Patient subsequently attended out-patients and remained well (18 months follow-up).

Code Nos. 24 - 30 not used.

31. E.G. Male. 30 yrs. Onset → test : 2½ yrs. F.H. doubtful - mother "odd, ? subnormal." At onset : puzzled, many bizarre delusions, clang associations ++, At test : delusions of

persecution, delusional perceptions. Thought broadcasting. Apathetic, reduced activity, aimless behaviour, little spontaneous speech. Off drugs, trifluoperazine → test : probably 6 months. Moderate response to phenothiazines, which he continued as an out-patient.

32. A.T. Male. 41 yrs. Onset → test : several months. F.H. -ve. Lack of interest, dull,apathetic, flattening of affect. Delusions of grandeur. No drugs prior to test. Slow response to phenothiazines. Remained in hospital throughout the 15 month follow-up.

33. E.R. Female. 39 yrs. Onset → test : 14 yrs. F.H. Mother mentally ill, probably a depressive illness. F.H.-ve for definite schizophrenia. Many previous admissions. At test : dull, apathetic, solitary, monosyllabic, admitted to hallucinations but would not detail these. Delusions about neighbours persecuting her. Off drugs (trifluoperazine) → test : 4 months. Fair response to Stelazine. Two subsequent readmissions with similar symptoms.

34. D.D. Female. 30 yrs. Onset → test : 12 yrs. F.H. -ve. Mute, inaccessible, mannerisms and stereotypies, forced grasping, Mitmachen and Mitgehen. A "manneristic catatonic." Off drugs (T) before test for 4 - 6 months. Poor response to phenothiazines. Transferred to a long-stay ward. (This patient

was admitted from a nursing home and had been in the state described for many months prior to admission).

35. W.C. Female. 32 yrs. Onset → test : 2-3 yrs. F.H. -ve. Prior admission to other hospitals. At test : manneristic, giggling, restless, thought disordered; thought insertion, delusions of persecution, auditorily hallucinated . Off drugs (thioridazine) → test : 3 months. Fair response to phenothiazines. Subsequently lost sight of.

36. This patient is also No. 22; he was tested both with methacetin and PCA.

37. J.W. Female. 28 yrs. Onset → test : 2 yrs. F.H. -ve. Insidious onset with ideas of reference, doubtful phonemes, hypochondriacal and anxious. At test : 2 months post partum. Excited. Hallucinations, chiefly auditory but a shadowy visual hallucination also occurred. Auditory hallucinations speaking of patient in third person. Passivity experiences, thought insertion and thought broadcasting. No drugs prior to test. Settled quickly on phenothiazines and discharged in one month. No subsequent symptoms (12 months follow-up, off drugs 6 months).

38. B.W. Female. 42 yrs. Onset → test : 4 yrs. F.H. -ve. At onset : agitated with abusive phonemes. Voices speaking about her in third person. Delusions of passivity. At test : 4 weeks post partum. Deluded, auditory hallucinations,

Gedankenlautwerden. Off drugs (C) → test : 3 weeks. Good response to phenothiazines. Remained well as out patient, (on drugs) during 12 month follow-up.

39. C.P. Male. 25 yrs. Onset → test : a few weeks. F.H. -ve. Lethargic, withdrawn, delusions of persecution, thought blocking. Peculiar behaviour, e.g. inserted pennies into his shoes. Flexibilitas cerea. No drugs prior to test. Excellent response to Haloperiodol. Not readmitted to the hospital where he was tested in 14 month follow-up.

40. T.H. Male. 22 yrs. Onset → test : 2 yrs. F.H. +ve, one brother firmly diagnosed as schizophrenic. Incoherent, deluded, (thought he had the secret of the cobalt bomb). Auditory hallucinations. Thought alienation, gross thought disorder. Drugs prior to test - a single dose of 10 mgm. haloperidol on previous day. Otherwise none for 4 months (before this chlorpromazine). Good response to phenothiazines. Attended out-patients, well and working (12 month follow-up).

41. W.A. Male. 19 yrs. Onset →test : several years. Insidious onset. F.H. -ve. Strange, withdrawn, apathetic, manneristic. Affect considerably flattened. Of poor intelligence, but with considerable deterioration in performance gradually over 4 yrs. Diagnosed as schizophrenia simplex. Off drugs (C) → test : probably three months. Poor response to phenothiazines. Transferred to a long-stay ward.

42. M.G. Male. 30 yrs. Onset → test : a few weeks. F.H. +ve : one sister firmly diagnosed as schizophrenic. Paternal grandmother "probably schizophrenic." Previous ulcerative colitis but no steroids and no other drugs for several months before test. E.S.M. school. At onset : grossly deluded, with some delusions of grandeur. Delusional perceptions. Somatic passivity experiences, overinclusive thinking. Good response to haloperidol. Discharged, but thereafter lost sight of.

43. C.J. Male. 17 yrs. Onset → test : several months. F.H. -ve. Admitted with an overdose of Artane; transferred to mental hospital after one week. Delusions of persecution, ideas of reference, thought disordered. Off drug (T) → test : 3 weeks. Fair response to phenothiazines. Remained well on drugs, (6 month follow-up).

44. P.G. Male. 53 yrs. Onset → test : 17 yrs. F.H. -ve. A number of admissions previously with delusions of persecution, hallucinations mainly auditory, passivity experiences. At test : deluded, auditorily hallucinated. Off drugs (trifluoperazine) prior to test : probably 2 - 3 yrs. Good response to phenothiazines. No readmission to hospital where tested in subsequent 12 months.

45. D.G. Male. 24 yrs. Onset → test : 6 yrs. F.H. +ve; brother definitely schizophrenic. Initial symptoms : panic feelings, bouts of depression. Later apathetic, depressed. Five years after onset : marked persecutory delusions. At test : thought disordered with word salad, delusions of persecution+++, passivity experiences, anxious, agitated. Off drugs (C) → test : 2 months. Good response to haloperidol. Two subsequent admissions with delusions of persecution during 12 month follow-up.

46. H.J. Male. 34 yrs. Onset → test : 9 months. F.H. maternal aunt apparently suffered delusions but no firm diagnosis of schizophrenia made. At test : systematised delusions of persecution (that the post office was framing him using concealed microphones). Auditory hallucinations doubtful - "a high pitched tone." Off drugs (Trifluoperazine) → test : probably 2 - 3 months. Good response to haloperidol. Discharged within one month, thereafter lost sight of.

47. B.F. Female. 28 yrs. Onset → test : 11 yrs. F.H. -ve, (although husband is firmly diagnosed as schizophrenic). At onset : mute, giggling, fatuous, manneristic, auditorily hallucinated. Many admissions up to (and after) time of test. Off drugs (C) → test : at least 3 months. At test : silly, thought disordered, thought blocking, thought broadcasting,

probably auditorily hallucinated. Fair response to phenothiazines.

48. E.H. Female. 64 yrs. Onset →test : a few weeks only. F.H.+ve, son firmly diagnosed as schizophrenic. At test : suspicious, delusions of persecution, gross thought disorder, neologisms, auditory hallucinations. No drugs prior to test. Excellent response to phenothiazines. Well at follow-up (8months) on drugs.

49. B.H. Female. 21 yrs. Onset → test : difficult to judge ? several yrs. F.H. -ve. Deteriorating work record; shallow emotional response noted by mother. Inconsiderate, apathetic. Diagnosed (by the late Professor F. J. Fish) as simple schizophrenia. No drugs prior to testing. Poor response to phenothiazines. Patient not subsequently readmitted but remained dull and affectively blunted.

50. D.P. Female. 29 yrs. Onset → test : a few weeks. F.H. -ve. Delusions of persecution centering chiefly around husband. Strange, silly behaviour, hypochondriacal complaints ++. Disorder of the experience of time, which "suddenly stopped and started a hundred times as fast." No drugs prior to test. Symptoms settled without treatment, but recurred in more florid form 3 months later, with delusions of persecution and abusive phonemes. Good response to phenothiazines.

51. D.K. Female. 22 yrs. Onset → test : 3 weeks. F.H. -ve.

Panic attacks, hallucinations, chiefly auditory. Inappropriate smiling and silly chatter, "posturing which she will not explain." No drugs prior to test. Good response to phenothiazines. Attending out-patients, well, (on drugs) at 9 month follow-up.

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