A STUDY OF SOME ASPECTS OF THE METABOLISM OF THE CATECHOLAMINES AND 5-HYDROXYTRYPTAMINE IN THE BRAIN.

Thesis

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SUMMARY

SUMMARY

1. An investigation of several aspects of the metabolism of the biogenic amines, dopamine, noradrenaline, and 5-hydroxytryptamine in the central nervous system, has been undertaken. The work was directed mainly to a quantitative study of the catabolites of the amines in human cerebrospinal fluid (c.s.f.) in order to monitor their turnover in the central nervous system in various clinical conditions. Of the catabolites considered, 3-methoxy-4-hydroxy-phenylacetic acid (homovanillic acid, HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC) derived from dopamine and 5-hydroxyindol-3-ylacetic acid (5-HIAA) from 5-hydroxytryptamine were specifically studied in relation to the therapy of parkinsonism with L-DOPA and the main cerebral catabolites of noradrenaline, 3-methoxy-4-hydroxyphenylethyleneglycol (MOPEG) and 3,4-dihydroxyphenylethyleneglycol (DOPEG), in relation to various states of mental derangement. The latter investigation involved the development of methodology for the estimation of MOPEG and DOPEG in c.s.f.

A short ancillary study was made of a possible role

of the choroid plexus in the removal of the acidic metabolites of the biogenic amines from c.s.f.

2. In parkinsonism, a concentration of DOPAC below normal levels was found in the lumbar c.s.f. of patients, an observation providing evidence, additional to that given by the previously observed lower concentrations of its other catabolite, HVA, of a decreased cerebral dopamine metabolism. A reduced concentration of 5-HIAA in such c.s.f. was confirmed.

There proved to be no correlation between the concentrations of these acid metabolites and the age or sex of the patients, the length of time they had shown parkinsonian symptoms or the severity of these symptoms.

3. The administration of L-DOPA (up to 8 g/day) to parkinsonian patients caused a dose-related increase in the concentrations of HVA and DOPAC in the lumbar c.s.f. but did not alter significantly the concentration of 5-HIAA. DOPA was only detectable in the c.s.f. when the dose of L-DOPA received by the patients was 3g or more per day.

Clinical improvement was associated only with the elevation of the HVA and DOPAC concentrations beyond threshold levels (250ng/ml for HVA and 15ng/ml for DOPAC) which were greatly in excess of the concentration of the substances in control c.s.f. (53ng/ml for HVA and 9ng/ml

for DOPAC). Above these threshold levels there was no correlation between the degree of improvement and the concentrations of HVA and DOPAC in lumbar c.s.f.

4. While relatively simple fluorimetric methods for the estimation of HVA, DOPAC and 5-HIAA are available, allowing studies of the turnover of their parent amines, no such method exists for the cerebral catabolites of noradrenaline, MOPEG and DOPEG.

The methods described in the literature which are of sufficient sensitivity for the estimation of MOPEG and DOPEG in brain or c.s.f. employ gas chromatography and electron capture detection. As the electron capture system is easily contaminated, these methods were considered unsuitable for the routine examination of these two glycols.

An attempt was made to develop a fluorimetric method for the estimation of MOPEG and DOPEG in c.s.f. which would be sufficiently sensitive yet simple enough for routine use.

5. A method depending on the formation of a fluorophor from MOPEG by oxidising it to vanillin with thiosemicarbazide was evolved but was found to be unreliable because, under the reaction conditions, quantitative oxidation of nanogram amounts of MOPEG to vanillin did not occur consistently.

A satisfactory method for the fluorimetric estimation of MOPEG depended on its oxidation with a ferric chloride reagent to produce a fluorophor with an activation wavelength of 325mμ and a fluorescence wavelength of 430mμ. The fluorescence intensity was proportional to the concentration of MOPEG in the original sample and the limit of sensitivity was about 25ng/ml. The reaction was, however, not specific to MOPEG and produced fluorophors with the same activation and fluorescence characteristics, but with different molar yields of fluorescence from a number of other O-methylated metabolites of the catecholamines.

6. A fluorophor was formed from DOPEG by condensing it with ethylene diamine. The fluorescence intensity was proportional to the concentration of DOPEG and the limit of sensitivity, in solution in water, was 4ng/ml. This reaction is not specific to DOPEG as fluorophor production occurs with several other compounds containing a catechol group.

7. The fluorophor-producing reactions for the estimation of MOPEG and DOPEG were not mutually interfering but were not specific for these compounds so that their prior separation from other similarly reacting catabolites of the catecholamines, which in the c.s.f. are particularly the acid derivatives, had to be effected in order to

obtain specificity.

Two methods were investigated.

a) The glycols were acetylated in alkaline aqueous medium and the derivatives separated from the acetylated acid catabolites by extraction into methylene dichloride. Direct estimation in the aqueous solution of the evaporated extract was possible but a high reagent blank decreased the sensitivity to an unsatisfactory degree. Suitable paper chromatography reduced the reagent blank and had the additional advantage of increasing the overall sensitivity of the analytical method by separating the two acetylated glycols and thus obviating the need to split the sample to allow their separate determinations.

In practice, however, this method was considered too complicated and time-consuming for its application to the routine estimation of the glycols in c.s.f. and a simpler technique was sought. In the development of this, attention was concentrated particularly on the separation and estimation of MOPEG, which is the major cerebral metabolite of noradrenaline.

b) The method finally used for the partial separation of MOPEG in samples of c.s.f., for routine estimations, involved its extraction into ethyl acetate and the further purification and separation from

O-methylated carboxylic acid derivatives of the catecholamines by chromatography of the aqueous solution of the evaporated extract on an anion exchange column.

This method is relatively simple in use and proved satisfactory for application to the routine estimation of MOPEG in c.s.f. Its applicability to the separation of DOPEG has not been investigated.

8. MOPEG exists in human c.s.f. as a conjugate, probably the sulphate ester. It was necessary to hydrolyse this ester prior to extraction and estimation of the glycol. Acid hydrolysis and enzymatic hydrolysis with the sulphatase-containing preparation 'Helicase' were investigated.

The conditions of low pH and elevated temperature which were required to hydrolyse a typical sulphate ester, p-nitrophenyl sulphate, were found to destroy free MOPEG. Acid hydrolysis was therefore considered unlikely to be suitable for the liberation of free MOPEG from its conjugate in samples of c.s.f.

Enzymatic hydrolysis was monitored using tritiated MOPEG conjugate which was obtained by biosynthesis from labelled normetanephrine injected into the cerebral ventricles of the rat and the conjugate subsequently isolated from the brains. Using an acetone powder of the commercially available sulphatase-containing preparation

'Helicase', suitable conditions were found for the quantitative enzymatic hydrolysis of MOPEG conjugate. Under these conditions the liberated MOPEG was not destroyed to any significant extent.

9. Enzymatic hydrolysis of the conjugate, extraction of the liberated glycol into ethyl acetate, chromatography on an anion exchange column and fluorophor production with the ferric chloride reagent, formed the basis of the method developed and used for the estimation of MOPEG in the c.s.f. of patients with various neurological disorders.

MOPEG has only been estimated in the c.s.f. of a small number of patients. The range of concentrations observed was large (17-170ng/ml) and there was no significant correlation between the concentration of the glycol and the clinical symptoms of these patients. It was observed, however, that the concentration of MOPEG in the c.s.f. of patients rated depressed tended to be lower than in patients rated manic.

10. Previous workers have found that homovanillic acid and 5-hydroxyindol-3-ylacetic acid, carboxylic acid catabolites of dopamine and 5-hydroxytryptamine present in the c.s.f., are removed into the blood by a transport system with an active component. The accumulation of radioactively labelled 5-HIAA by the isolated choroid

plexuses of the rabbit was studied in order to determine whether this tissue could be responsible for the removal of the acid from the c.s.f.

Choroid plexuses of the rabbit accumulated 5-HIAA, *in vitro*, to give a concentration in the tissue up to eight times that in the medium. The choroid plexuses from the fourth ventricle accumulated 5-HIAA to a greater extent than those from the lateral ventricles. The accumulation process was dependent on the formation of ATP in the tissue, as it was inhibited by the presence of 2,4-dinitrophenol in the incubation medium. The accumulation was also inhibited by the presence in the incubation medium of HVA or probenecid, substances which have been shown to inhibit the transport of 5-HIAA *in vivo*.

GENERAL INTRODUCTION.

GENERAL INTRODUCTION

Since the first identification in brain of the biogenic amines, noradrenaline, adrenaline (Vogt, 1954), dopamine (Carlsson,1959) and 5-hydroxytryptamine (Amin, Crawford and Gaddum, 1953) many workers have studied the effects of drugs and/or environmental changes on their metabolism. The metabolites of dopamine, 3,4-dihydroxyphenylacetic acid and 3-methoxy-4-hydroxyphenylacetic acid (homovanillic acid) have been estimated in brain and cerebrospinal fluid (Rosengren,1960; Andén, Roos and Werdinius,1963a; Andén, Roos and Werdinius,1963b; Sharman, 1963; Ashcroft, Crawford, Dow and Guldberg,1968) as has the metabolite of 5-hydroxytryptamine, 5-hydroxyindol-3-ylacetic acid (Ashcroft and Sharman,1960). Attempts to find an acid metabolite of noradrenaline in the central nervous system have, however, failed except for one report (Matsuoka, Yoshida and Imaizumi,1964) of the presence of 3,4-dihydroxymandelic acid in the brain of rabbits. Neutral metabolites of noradrenaline, 3-methoxy-4-hydroxyphenylethyleneglycol and 3,4-dihydroxyphenylethyleneglycol have been shown to exist in brain and cerebrospinal fluid (Schanberg, Breese, Schildkraut, Gordon and Kopin,1968; Sharman,1969). In most species of animals examined, these glycols are present as their sulphate esters; in some however they exist as the free alcohols.

Alterations in the <u>in vitro</u> metabolism of the biogenic amines can be studied in a number of ways. One involves the administration

to the animal of either the radioactively labelled amine or one of its precursors. After a given time the labelled metabolites of the amines are separated, identified and estimated. This method does not involve the metabolism of the endogenous amine but rather the metabolism of the labelled amine. It has thus to be assumed that the administered amine becomes distributed throughout the brain in the same way as the endogenous amine. This is not necessarily the case and metabolism may occur at sites not normally accessible to the amine.

Estimation of the endogenous concentrations of the amines and/or their metabolites within the central nervous system can also be used to indicate changes in metabolism. In experimental animals this can be done in brain tissue but when dealing with man, brain samples can only be obtained post-mortem or by biopsy during surgery. Both these are unsatisfactory, the post-mortem tissue because changes may occur on death and the biopsy specimen because, of necessity, it can only be very small. The closest approach which can normally be made to the central nervous system of man is the cerebrospinal fluid. The amines themselves are not present in measurable quantities in the cerebrospinal fluid but the metabolites of dopamine, noradrenaline and 5-hydroxytryptamine have all been shown to be present (Ashcroft and Sharman, 1960; Andén et al., 1963b; Schanberg, Breese, Schildkraut, Gordon and Kopin, 1968). That changes in the concentration of the metabolites in the cerebrospinal fluid parallel changes in the concentration of the amines and their metabolites in the brain has been postulated for the dopamine metabolite, homovanillic

acid, by Guldberg, Ashcroft, Turner and Hanieh (1969). In the first section of this thesis the metabolites of dopamine, 3,4-dihydroxyphenylacetic acid and homovanillic acid, and the 5-hydroxytryptamine metabolite, 5-hydroxyindol-3-ylacetic acid have been estimated in the lumbar cerebrospinal fluid of parkinsonian patients both before and during treatment with an amino acid precursor of dopamine, 3,4-dihydroxyphenylalanine, and an attempt has been made to correlate these concentrations with the clinical condition of the patients.

The second section of this thesis describes the development of a method for the estimation in cerebrospinal fluid, of 3-methoxy-4-hydroxyphenylethyleneglycol, the major cerebral metabolite of noradrenaline (Schanberg, Schildkraut, Breese and Kopin, 1968). Methods for the estimation of this compound are described in the literature (Ruthven and Sandler, 1965; Wilk, Gitlow, Clarke and Paley, 1967; Schanberg, Breese, Schildkraut, Gordon and Kopin, 1968; Calne, Karoum, Ruthven and Sandler, 1969a; Sharman, 1969) but the majority of these are unsuitable for routine estimations either due to lack of sensitivity or because they are too complicated and time consuming. The object of the work presented in this section was to develop a simple but sensitive method for the routine estimation of 3-methoxy-4-hydroxyphenylethyleneglycol in cerebrospinal fluid.

The metabolites of the biogenic amines which are present in cerebrospinal fluid are removed from there into the blood. Ashcroft, Dow and Moir (1968) have shown that the acid metabolite of

5-hydroxytryptamine, 5-hydroxyindol-3-ylacetic acid, is transported out of the cerebral ventricles by a mechanism, additional to bulk absorption, which displays the characteristics of an 'active transport' system. The structures in the brain which have been implicated in this transport process are the highly vascular choroid plexuses. In the third section of this thesis the accumulation of 5-hydroxyindol-3-ylacetic acid by the isolated choroid plexus has been studied in order to determine whether this structure could be involved in the active transport of 5-hydroxyindol-3-ylacetic acid from the cerebrospinal fluid to the blood.

SECTION 1

Phenolic acid concentrations in the lumbar cerebrospinal fluid of parkinsonian patients treated with L-DOPA.

INTRODUCTION

The existence of lower concentrations of dopamine in the caudate nucleus and substantia nigra in parkinsonian patients than in a control group (Ehringer and Hornykiewicz, 1960; Hornykiewicz, 1962) has led to the suggestion that a change in the metabolism of this amine is associated with the syndrome. This concept is supported by the finding that the urinary concentration of dopamine in these patients is also reduced (Barbeau, Murphy and Sourkes, 1961; Bischoff and Torres, 1962). The main metabolite of dopamine, homovanillic acid (HVA), is present in reduced concentrations in the brain (Bernheimer and Hornykiewicz, 1964) and in the c.s.f. (Bernheimer, Birkmayer, Hornykiewicz, Jellinger and Seitelberger, 1966; Guldberg, Turner, Hanieh, Ashcroft, Crawford, Perry and Gillingham, 1967; Johansson and Roos, 1967; Guldberg, Ashcroft, Turner and Hanieh, 1969) of parkinsonian patients, but its urinary excretion is not altered (Geer and Williams, 1963; Calne, Karoum, Ruthven and Sandler, 1969a), suggesting the absence of any general alteration in dopamine metabolism being associated with this syndrome.

The concentrations of other biogenic amines, namely noradrenaline and 5-hydroxytryptamine (5-HT) are also reduced in several areas of brain obtained on autopsy from parkinsonian patients (Bernheimer, Birkmayer and Hornykiewicz, 1961; Hornykiewicz, 1962),

but the reduction in the concentration of these two amines in the caudate nucleus and putamen of between 55% and 65% is less than the decrease in concentration of 85% to 90% observed for dopamine in the same areas. The excretion of 5-hydroxytryptamine and noradrenaline in the urine of parkinsonian patients does not differ from those in control subjects (Barbeau et al., 1961; Resnick, Gray, Koch and Timberlake, 1962). The concentration of 5-hydroxyindol-3-ylacetic acid (5-HIAA), the main metabolite of 5-hydroxytryptamine, is lower in the c.s.f. of parkinsonian patients than in a control group (Guldberg et al., 1967; Johansson and Roos, 1967; Guldberg et al., 1969) and its concentration in the urine has been reported to be significantly reduced in parkinsonism (Barbeau and Jasmin, 1961). This finding, however, was not confirmed by Resnick et al., (1962) who detected no significant difference in the concentration of 5-HIAA in the urine of parkinsonian and control patients either before or after receiving the 5-HT precursor, 5-hydroxytryptophan.

In view of a possible aetiological significance of the lowered cerebral dopamine levels in this syndrome, Birkmayer and Hornykiewicz (1961) treated parkinsonian patients with intravenous doses of 50 mg to 150 mg of the dopamine precursor, L-3,4-dihydroxyphenylalanine (L-DOPA) and obtained a reduction in akinetic symptoms. This was substantiated by Barbeau (1962) who gave parkinsonian patients oral doses of 200 mg of L-DOPA. This author also showed that D-DOPA when given orally had no effect on the symptoms of parkinsonism, and it was later found that this isomer

of DOPA would not pass from the blood into the brain of rats (Gey and Pletscher, 1964). In a further study on the effect of DOPA administration to parkinsonian patients, McGeer and Zeldowicz (1964) gave D,L-DOPA orally in doses of 1 g to 5g per day or intravenously in doses of 0.2 g to 0.5 g per day but did not obtain sufficient improvement for them to consider DOPA a useful therapeutic agent. Cotzias, Papavasiliou, Gellene, Aronson and Mena (1969) reported that the oral administration of L-DOPA in doses up to 8 g per day produced remission of bradykinesia to a varying extent in every parkinsonian patient thus treated. In order to minimise adverse side effects, these authors "titrated" the parkinsonian patients with DOPA.

In a similar trial, Calne et al., (1969a) studied the changes in concentration of the urinary metabolites of L-DOPA when this drug was administered orally to parkinsonian patients in doses of 0.5 g to 2.5 g per day and found that large quantities of the dopamine metabolites, HVA and 3,4-dihydroxyphenylacetic acid were excreted. However, as these authors point out, the concentrations of the dopamine metabolites in the c.s.f. are likely to reflect the metabolism of DOPA in the brain more faithfully than do urinary levels. In the present study, DOPA and the acid metabolites of dopamine and 5-hydroxytryptamine have been estimated in the lumbar c.s.f. of parkinsonian patients prior to and during treatment with L-DOPA. An attempt has been made to correlate the biochemical data with the pretreatment clinical assessment of the patients, and

with the degree of improvement observed on administration of the drug.

BIOCHEMICAL INVESTIGATIONS

Parkinsonian Patients

The patients in this study were admitted to the Western General Hospital's Surgical Neurology Ward (S.N.3). They showed as their major symptoms those which are known to be little affected by surgery; in particular bradykinesis, and over half the cases had previously undergone steriotactic operation for the relief of tremor and rigidity (Gillingham, Watson, Donaldson and Naughton, 1960). Prior to the administration of L-3,4-dihydroxyphenylalanine (L-DOPA) all other drug treatment was stopped and each patient was rated on a three point scale for rigidity, tremor, bradykinesia, speech volume and dementia, cine recordings made and, after treatment, the degree of improvement noted.

Administration of L-DOPA

The L-DOPA used was supplied by Koch-Light Co., Ltd., and contained not less than 99% L-DOPA.

The patients were given L-DOPA orally, starting at a low dose (500 mg three times a day) which was increased in gradual steps. The length of time a patient remained on any one dose level and also the final maintenance dose were determined by the tolerance of the patient to the drug. In no case was the dose increased above

8 g per day.

In some patients, L-DOPA caused vomiting and nausea. With several of these patients such side effects could be avoided by lowering the administered dose followed by increasing it again more gradually, in others it was found necessary to stop treatment with L-DOPA.

Removal and Storage of Lumbar c.s.f.

Approximately 10 ml of lumbar cerebrospinal fluid (c.s.f.) was withdrawn from the patients before the start of treatment with L-DOPA and again when the maintenance dose was reached. This was done as far as possible at a constant time after the patient received the drug. In those patients where the drug had to be terminated, the second withdrawal of the lumbar fluid was carried out immediately after discontinuing L-DOPA.

The c.s.f. was collected in a clean glass-stoppered 10 ml graduated test tube and was stored in the freezing compartment of a domestic refrigerator until it was collected from the ward (S.N.3) and placed at -20°C in the laboratory. The c.s.f. was always analysed within one week of withdrawal.

Control Patients

Six patients undergoing myelography at the Northern General Hospital, Edinburgh, had 5 ml of lumbar c.s.f. withdrawn. These c.s.f. samples were stored as previously described and were used for

the estimation of control levels of 3,4-dihydroxyphenylacetic acid (DOPAC).

BIOCHEMICAL ANALYSES

Materials

Homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC) were obtained from Calbiochem and 5-hydroxyindol-3-ylacetic acid (5-HIAA) from Koch-Light Co., Ltd.

All other reagents were Analar grade except for the ammonia solution which was Aristar grade (British Drug Houses) and the hydrochloric acid which was Microanalytical Reagent (B.D.H.) grade. The ethylenediamine used in the estimation of DOPAC and DOPA was of Laboratory Reagent (B.D.H.) grade and was distilled twice and stored at -20°C until required.

The ethyl acetate used for the extraction of the phenolic acids was from a 500 ml bottle which was opened on the same day as the analysis was to be carried out. Deionized distilled water was used throughout and all extractions and reactions involved in the estimations were carried out in glass tubes fitted with glass stoppers.

The buffers used were prepared as follows:-

Borate buffer 0.1M boric acid adjusted to pH 8.6 with 0.1M sodium hydroxide.

Phosphate buffer 0.1M sodium dihydrogen orthophosphate adjusted to pH 7.4 with 0.1M disodium hydrogen orthophosphate.

Tris buffer 0.1M 2-amino-2-(hydroxymethyl) propane-1: 3-diol adjusted to pH 8.6 with conc. HCl.

A glass electrode was used for all pH measurements.

Standard solutions Solutions containing 1 mg/ml of HVA, DOPAC, 5-HIAA or DOPA in water were stored at 4°C for periods up to three weeks up to which time there was found to be no detectable destruction of these substances. These standard solutions were used to prepare all standard curves and recoveries.

Fluorimetry All fluorescence measurementswere carried out in an Aminco-Bowman Spectrophotofluorimeter with slit arrangement No. 5 (Aminco-Bowman manual). The solution containing the fluorophor was placed in a 3 ml Spectrosil (Scientific Supplies) cell with an internal cross section of 10 mm square. A minimum volume of 1.2 ml of liquid was required in order to prevent the meniscus interfering with the fluorescence measurements.

Extraction of Phenolic Acids from c.s.f.

The concentrations of the dopamine metabolites, 3,4-dihydroxy-phenylacetic acid (homovanillic acid, HVA), and of the 5-hydroxy-tryptamine metabolite, 5-hydroxyindol-3-ylacetic acid (5-HIAA) were estimated in the c.s.f. essentially according to the methods described by Ashcroft, Crawford, Dow and Guldberg (1968).

The c.s.f. sample was thawed, measured with a graduated pipette into a glass stoppered test tube of 80 ml capacity and

diluted to 10 ml with water. The mixture was saturated with sodium chloride (approximately 4 g), acidified to pH 1-2 with 50 µl of concentrated HCl and the phenolic acids were extracted into ethyl acetate by shaking for 5 minutes with 20.0 ml of the solvent. The mixture was centrifuged at 3,000 rev/min for 5 minutes in order to separate the two phases. After 19.0 ml of the upper organic layer had been removed, the extraction was repeated and a further 20.0 ml of the organic layer was pooled with that from the first extraction.

For the estimation of HVA, 10.0 ml of the pooled ethyl acetate extract was shaken for 5 minutes with 1.4 ml of sodium borate buffer pH 8.6. The two phases were separated by centrifugation at 3,000 rev/min and 1.0 ml of the lower aqueous layer transferred to a 15 ml glass-stoppered test tube.

The remaining 29.0 ml of the ethyl acetate extract was shaken for 5 minutes with 2.4 ml 0.1M phosphate buffer pH 7.4 and the mixture then centrifuged at 3,000 rev/min for 5 minutes. Two portions, each of 1.0 ml, of the lower phosphate buffer phase were transferred to separate 15 ml glass-stoppered test tubes for the estimation of DOPAC and 5-HIAA.

Estimation of HVA The method used for the estimation of HVA was essentially that of Anden, Roos and Werdinius (1963).

To 1.0 ml of the borate buffer extract in a 15 ml test tube was added 1.0 ml 5M ammonia and 0.2 ml of a 10 mg% w/v solution of potassium ferricyanide. After exactly 4 mins the oxidation was

terminated by the addition of 0.2 ml of a 100 mg% w/v solution of cysteine. The fluorophor thus obtained from HVA had an activation wavelength of 325 mμ and a fluorescence wavelength of 430 mμ (uncorrected) and its concentration was estimated within 30 mins from meter readings with the activation and fluorescence wavelengths of the fluorimeter set to correspond to those of the fluorophor.

Estimation of DOPAC The method of Rosengren (1960) was used for the estimation of this phenolic acid.

To 1.0 ml of the phosphate buffer extract in a 15 ml glass-stoppered test tube, was added 0.5 ml of an ethylenediamine/ ammonium chloride mixture (one part of twice distilled ethylenediamine to 1.3 parts of 4M ammonium chloride, by volume). The resulting solution was heated, with shaking, in a metabolic shaking waterbath at 50°C for 20 mins in the dark, and then rapidly cooled to room temperature. The fluorophor produced was kept in the dark until its concentration was estimated, within 30 mins of fluorophor production, from a scan of its activation spectrum. The fluorophor had an activation wavelength of 420 mμ and a fluorescence wavelength of 530 mμ (uncorrected).

Estimation of 5-HIAA The method used was as described by Ashcroft and Sharman (1962).

The phosphate buffer extract (1.0 ml) was acidified by the addition of 0.4 ml conc HCl containing 50 mg% w/v ascorbic acid.

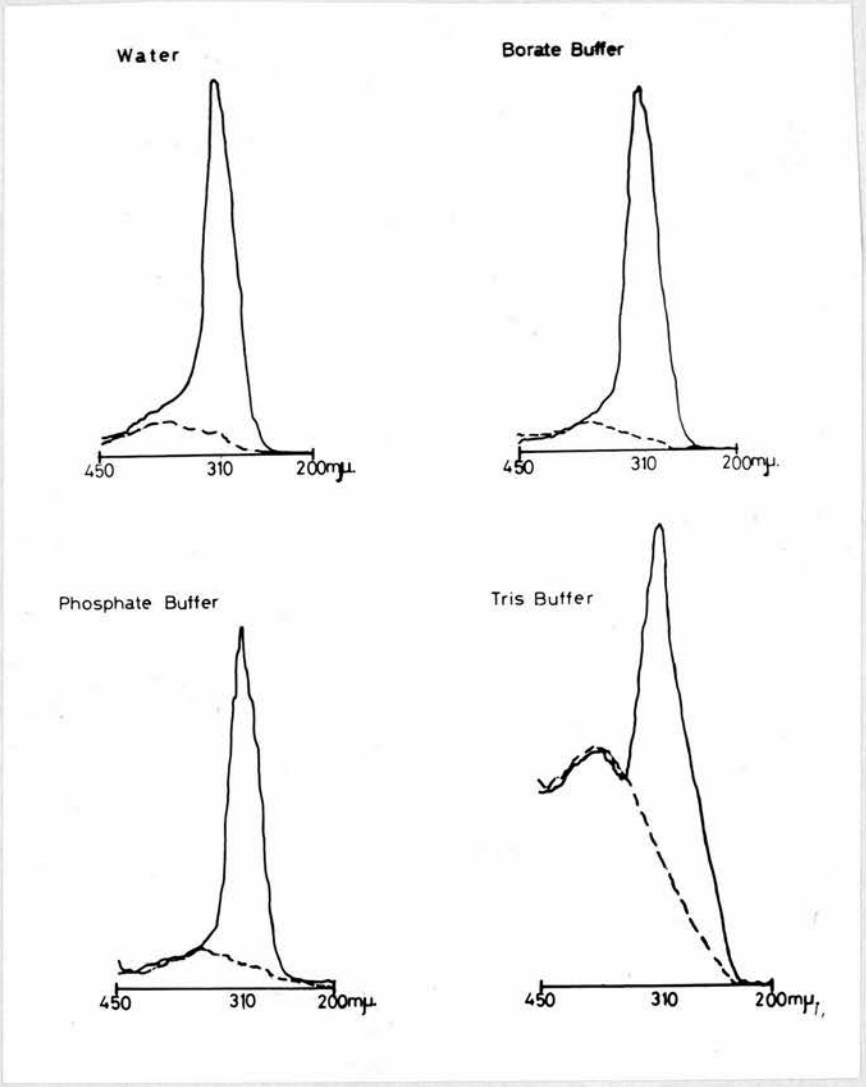


Fig. 1:1

Activation spectrum of the fluorophor from 100 ng 5-hydroxyindol-3-ylacetic acid (5-HIAA) in 1 ml water, borate buffer pH 8.6, phosphate buffer pH 7.4 tris buffer pH 8.6 formed on the addition of 0.4 ml conc HCl containing 50 mg% w/v ascorbic acid.

———— = Activation scan of 5-HIAA fluorophor

- - - - - - - - = Activation scan of 1 ml of the solvent containing 0.4 ml conc HCl/ascorbic acid

The fluorescence monochromator was set at 550 mu (uncorrected).

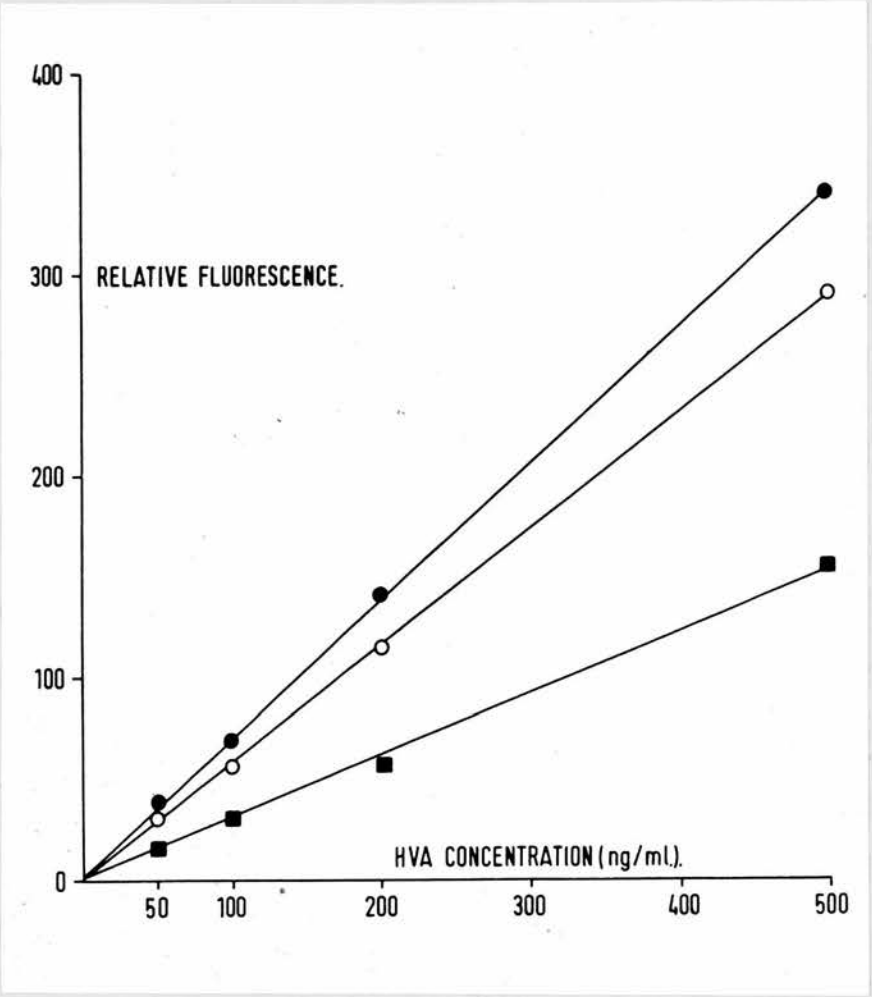


Fig. 1:2

The relationship between the concentration of homovanillic acid (HVA) and the relative fluorescence of the fluorophor produced from it in water (●—●), borate buffer pH 8.6 (○—○) and phosphate buffer pH 7.4 (■—■). The fluorescence of the reagent "blank" has been substracted in each case.

The intensity of the fluorescence derived from the 5-hydroxyindolic group was immediately estimated at an activation wavelength of 310 mμ and a fluorescence wavelength of 550 mμ. A yellow filter with a lower wavelength cut off of 420 mμ was placed between the fluorescence monochromator and the photomultiplier. This prevented a secondary scatter peak at 275 mμ from interfering with the specific peak at 310 mμ when the activation spectrum was recorded.

Choice of buffers for the back extraction of the phenolic acids

The buffer used by Ashcroft et al., (1968) in the estimation of HVA, DOPAC and 5-HIAA in the ventricular c.s.f. of dogs was 0.1M tris-hydroxymethylaminomethane (2-amino-2-(hydroxymethyl) propane-1:3 diol) buffer pH 8.6. This buffer, however, was found to give high "blank" readings in the estimation of these acids, thus reducing the overall sensitivity of the methods (taken as being the quantity of substance required to produce a fluorescence equal to that of the blank). It was therefore decided to try to find a more suitable buffer system.

Borate and phosphate buffers gave lower blank readings in the estimations of HVA, DOPAC (Table 1:1) and 5-HIAA (Fig. 1:1) than did tris buffer, but phosphate buffer caused quenching of the fluorophor produced from HVA (Fig. 1:2). Borate buffer did not cause such a high degree of quenching of the HVA fluorophor and it was decided to use it in the estimation of this acid. Neither phosphate nor borate buffer caused quenching of the DOPAC or 5-HIAA

TABLE 1:1

Relative fluorescence values for 1.0 ml of water, borate buffer pH 8.6, phosphate buffer pH 7.4, and tris buffer pH 8.6 taken through the procedures for the production of the fluorophors from homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC).

	Relative fluorescence	
	HVA	DOPAC
Water	33	3
Borate buffer pH 8.6	55.5	5
Phosphate buffer pH 7.4	45	5
Tris buffer pH 8.6	111	10

fluorophors, but phosphate buffer was preferred in the estimation of these two acids because it was considered that its lower pH might afford a degree of protection against oxidation of these substances. This point has since been investigated and borate buffer, despite its higher pH, was found to give the same results as phosphate buffer when used in the estimation of DOPAC and 5-HIAA.

Estimation of DOPA As DOPA is an amino acid it was not extracted from the acidified c.s.f. by the ethyl acetate.

The salt saturated aqueous layer remaining after the extraction of the phenolic acids with ethyl acetate, was kept at -20°C for not less than 1 hr, in order to reduce its salt content, and approximately 5 ml then decanted into a clean 30 ml test tube. Any residual ethyl acetate was distilled off at room temperature under reduced pressure, care being taken to remove as little water as possible. A portion, 1.0 ml, of the residual solution was transferred to a 15 ml glass-stoppered test tube and 0.5 ml of the ethylenediamine/ammonium chloride mixture used for the estimation of DOPAC, added to it. The mixture was heated, with shaking, at 50°C for 20 mins in the dark, in a waterbath, cooled rapidly to room temperature and the fluorophor produced from DOPA estimated from a scan of its activation spectrum. The concentration of the fluorophor was estimated within 30 mins of its production and it had an activation wavelength of 360 mμ and a fluorescence wavelength of 470 mμ (uncorrected values).

Recoveries and Blanks In order to determine the recovery of the HVA, DOPAC, 5-HIAA and DOPA through the procedure, 200 ng of HVA, DOPAC and 5-HIAA and 1 µg of DOPA were added to 10 ml water and were extracted in the same way as from the c.s.f. Ideally recoveries from the c.s.f. should have been used but it was found that the entire 10 ml of c.s.f. from the patients was required for the estimation of the acids.

'Extraction blanks' were obtained by processing 10 ml of water through the extraction procedure and treating the appropriate buffer extracts with the fluorophor-producing reagents.

Evidence for the Identity of the Substances Estimated Fluorimetrically

Evidence for the identity of the substances estimated in the c.s.f. was obtained from the fluorescence characteristics of the fluorophors produced from them. These fluorophors had the same activation and fluorescence wavelengths as the fluorophors from authentic HVA, DOPAC, 5-HIAA and DOPA.

In order to confirm the identity of these substances, four of the c.s.f. samples, known to be from patients on a large dose of L-DOPA were examined chromatographically. The samples were divided into two equal portions, the acids in one portion were estimated as before whilst those from the other were extracted, after salt saturation and acidification, into 10 ml ethyl acetate. After centrifugation to separate the phases, 9.0 ml of the top

ethyl acetate layer was transferred to a 100 ml round bottomed flask, the extraction repeated and a further 10.0 ml of the organic layer pooled with that from the first extraction. The extract was evaporated to dryness at room temperature under a stream of nitrogen. The residue was dissolved in 0.3 ml of methanol and transferred by replicate applications to a small spot on a pre-coated Silica Gel thin layer chromatographic plate, 0.25 mm thick (Merck). The flask was rinsed with a further 0.3 ml methanol which was applied to the same spot. Markers, approximately 10 µg of HVA, DOPAC, 5-HIAA, vanillinmandelic acid (VMA) and 3,4-dihydroxymandelic acid (DOMA) were applied to the plate which was developed in the lower phase of a mixture of chloroform: acetic acid: water (2:2:1 by vol) (Ashcroft et al., 1968). When the solvent had reached the top of the plate, the plate was removed from the tank, placed in a stream of nitrogen for 5 mins and then returned to the tank for a second development. When the solvent had again reached the top of the plate, the plate was taken from the tank and most of the developing solvent removed by placing the plate in a dry "Chromatank" (Shandon) through which was passed a brisk stream of nitrogen for 1 hr. The areas of the plate containing the c.s.f. extracts were covered with glass while the marker acids were visualised with diazotised p-nitroaniline.

The spray used for the visualisation consisted of 20 ml of a saturated solution of p-nitroaniline in 0.1N HCl to which had been added 1.2 ml of 5% (w/v) sodium nitrite just before use. The plate

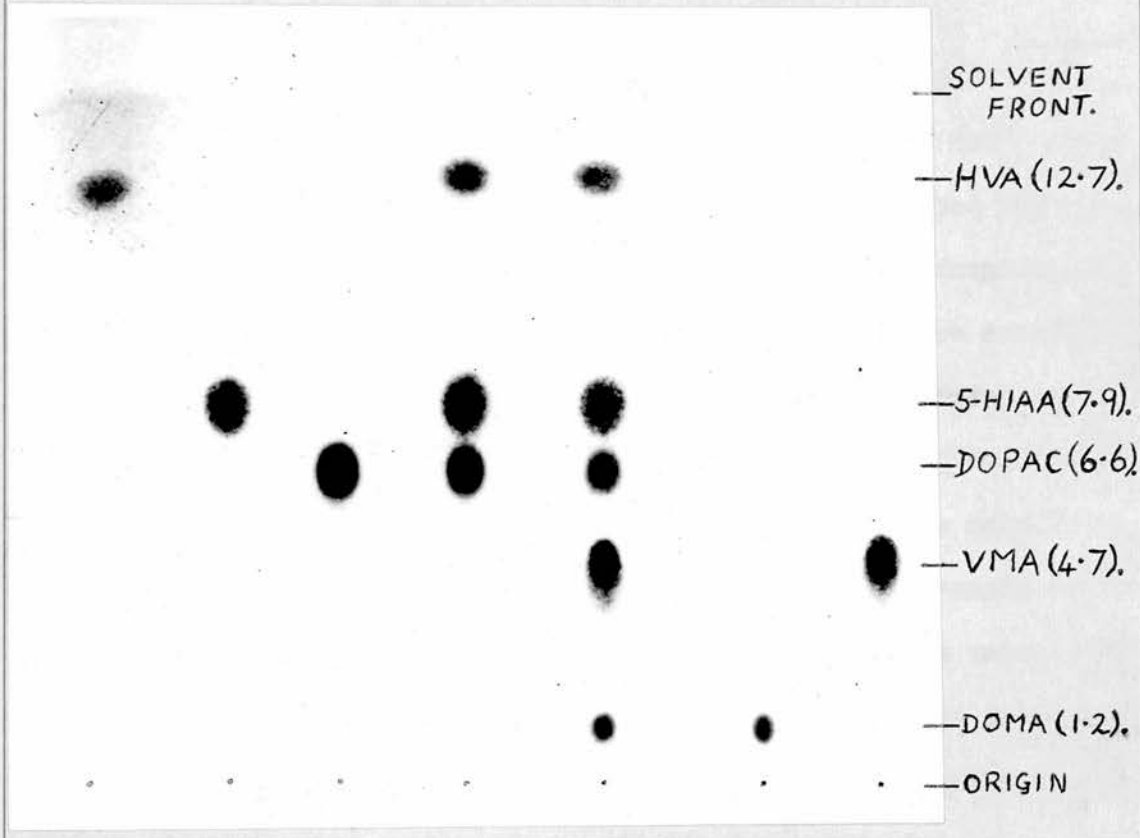


Fig. 1:3

Separation of 3,4-dihydroxymandelic acid (DOMA), 3-methoxy-4-hydroxymandelic acid (VMA), 3,4-dihydroxyphenylacetic acid (DOPAC), 3-methoxy-4-hydroxyphenylacetic acid (homovanillic acid, HVA) and 5-hydroxyindol-3-ylacetic acid (5-HIAA) on a precoated silica gel thin layer chromatographic plate (Merck) developed twice in the lower phase of a mixture of chloroform: acetic acid: water (2:2:1 by volume). The numbers in brackets are the distances in centimetres of the acids from the origin.

was sprayed with this solution, allowed to dry and then sprayed with 5% (w/v) sodium hydroxide in 50% (w/v) methanol. The diazotised p-nitroaniline, in alkaline solution, couples with phenols to produce red to purple azo-compounds.

The parts of the thin layer adsorbent of the c.s.f. extract chromatograms, corresponding to the marker HVA, DOPAC and 5-HIAA positions were scraped off and eluted by shaking the adsorbent with 1.0 ml water in a 2 ml conical polystyrene test tube for 30 mins. The silica gel was sedimented by centrifugation at 15,000 rev/min for 2 mins and 0.8 ml of the supernatant fluid transferred to a 15 ml glass-stoppered test tube. The eluate was diluted to 1.0 ml with water and the concentration of the appropriate phenolic acid estimated as previously described.

The separation of the phenolic acids obtained by chromatography on a precoated silica gel thin layer chromatographic plate (Merck) developed twice in the lower phase of a mixture of chloroform: acetic acid: water (2:2:1 by vol) is shown in Fig. 1:3.

The percentage recovery from 5.0 ml water of 1 or 2 µg of HVA and 100 or 200 ng of DOPAC when extracted and chromatographed were 60 ± 4 and 47 ± 4 (mean $\pm$ standard deviation, n = 8) respectively. There was good agreement between the concentrations of these two acids in the lumbar c.s.f. of parkinsonian patients receiving L-DOPA, when they were estimated by the chromatographic or by the simple extraction method (Table 1:2).

5-HIAA was not detectable when 100 or 200 ng were extracted into

TABLE 1:2

The concentration (ng/ml c.s.f.) of homovanillic acid (HVA), 3, 4-dihydroxyphenylacetic acid (DOPAC) and 5-hydroxyindol-3-ylacetic acid (5-HIAA) in the lumbar c.s.f. of 4 patients receiving L-DOPA.

Acids	Method of Estimation	1	2	3	4
HVA	Chromatography	510	544	324	715
	Extraction	488	500	340	689
DOPAC	Chromatography	59	46	31	60
	Extraction	54	50	34	55
5-HIAA	Chromatography	-	-	-	-
	Extraction	11	22	14	12
Dose of L-DOPA (g/day)		6	6	4.5	8

ethyl acetate, chromatographed and the appropriate part of the thin layer plate eluted in water, nor could it be detected when the same quantity of 5-HIAA was applied directly to the plate, chromatographed and eluted. This was thought to be due to adsorption of the 5-HIAA onto the silica gel. Elution with 0.1M HCl or 0.1M NH_4 OH also failed to remove the 5-HIAA from the adsorbent.

RESULTS

The clinical and biochemical results are summarised in Table 1:3. For various reasons it was not possible to obtain both "pre" and "on-treatment" samples of c.s.f. from all 31 patients examined, nor did it prove possible to carry out a complete analysis on every sample.

"Pre-treatment studies"

The estimated concentrations of HVA, DOPAC and 5-HIAA in the lumbar c.s.f. of the parkinsonian patients before treatment with L-DOPA are given in Table 1:4, which shows, for comparison, the concentrations found by Guldberg et al., (1969) in control subjects.

In c.s.f., the limit of sensitivity of the DOPA estimation was approximately 50 ng/ml of c.s.f. The "blank" fluorescence, however, varied from sample to sample and DOPA was considered to be undetectable if a fluorescence maximum was not obtained at 360 mμ when the activation spectrum was recorded from 200 mμ - 450 mμ;

TABLE 1:3

The concentration (ng/ml) of homovanillic acid (HVA), 3,4-dihydroxyphenylacetic acid (DOPAC) and 5-hydroxyindol-3-ylacetic acid (5-HIAA) in the lumbar c.s.f. of parkinsonian patients both before and during treatment with L-DOPA.

The patients are listed in order of increasing dose of L-DOPA.

Explanatory notes:-

"Duration of illness" in years

"Operation" - R = right side
L = left side
B = bilateral
No = no operation

"Pre-treatment rating" - graded 0 to ++ according to severity - "Total" = total of pre-treatment ratings.

"Improvement" - improvement on receiving L-DOPA graded from 0 to ++ according to degree.

* blood in sample

"_____" in table indicates that no information was available about this parameter in this particular case.

Patient	Sex	Age	Duration of illness	Operation	Upper line pretreatment Lower line on treatment concentrations (ng/ml) of HVA	DOPAC	5-HIAA	c.s.f. DOPA (ng/ml)	Dose of L-DOPA (g/day)	Rigidity	Tremor	Bradykinesia	Speech volume	Dementia	Total	Improvement
1	M	60	11	L	27	4	14									
					-	-	-	-	1.5	+	+	+	+	+	5	0
2	F	56	6	R	32	2	17									
					-	-	-	-	1.5	+	+	+	0	0	3	0
3	F	58	16	B	-	-	-									
					126	-	-	-	1.5	+	+	++	+	+	6	0
4	F	-	-	-	-	-	-									
		-			116	10	10	<50	1.5	-	-	-	-	-	-	0
5	F	62	7	no	8	9	8									
					103	11	13	<50	1.5	+	+	+	0	+	4	0
6	M	48	14	no	-	-	-									
					46	-	-	-	3	+	+	++	+	+	6	0
7	M	55	4	L	12	10	-									
					-	25	17	-	3	+	+	+	+	+	5	+
8	M	61	10	L	12	-	22									
					263*	84*	16*	138*	3	+	+	+	+	++	6	++
9	M	63	4	no	10	-	-									
					307	22	10	132	3	+	+	+	+	+	5	++
10	M	73	20+	no	19	4	11									
					-	-	-	-	3	+	+	+	+	+	5	0
3	F	58	16	B	-	-	-									
					617	48	23	305	3	+	+	++	+	+	6	++
11	F	67	20	B	18	8	14									
					570	16	17	115	3	+	+	+	+	0	4	++
12	M	37	5	L	8	4	9									
					168	15	17	-	4.5	+	+	+	+	0	4	+
13	M	45	4	no	24	5	11									
					305	24	13	106	4.5	+	+	+	+	++	6	+
14	F	66	4	no	29	6	18									
					-	35	22	-	4.5	+	+	+	0	0	3	+
15	-	-	-	-	17	-	12									
	-				317	27	12	-	4.5	+	+	+	+	0	4	+
16	M	49	20	B	14	4	10									
					277	16	10	136	6	+	+	+	+	0	4	+
17	M	59	3	no	23	-	-									
					277	-	-	-	6	+	+	+	+	0	4	+
18	M	68	16	R	14	8	9									
					478	43	14	-	6	+	+	+	+	0	4	++
19	F	61	13	B	12	8	13									
					-	-	-	-	6	+	+	+	+	+	5	0
20	F	-	-	-	-	7	19									
	-				413	19	16	-	6	-	-	-	-	-	-	++
6	M	48	14	no	-	-	-									
					618	-	-	-	8	+	+	++	+	+	6	++
21	M	55	6	L	22	8	8									
					538	56	-	-	8	+	+	+	+	0	4	+
22	M	61	6	no	6	3	6									
					609	58	14	317	8	+	+	+	0	0	3	++
23	M	72	8	L	21	6	14									
					1755	147	14	-	8	+	+	++	++	+	7	++
24	F	32	6	no	24	9	19									
					742	21	19	239	8	++	+	++	+	0	6	++
25	M	50	15	B	10	-	7									
					-	-	-	-	-	+	+	+	++	0	5	0
26	M	59	3	no	7	3	8									
					-	-	-	-	-	+	+	+	+	0	4	+
27	M	61	8	R	8	8	7									
					-	-	-	-	-	+	+	+	0	0	3	+
28	F	52	5	no	12	12	11									
					-	-	-	-	-	+	+	+	0	0	3	+
29	F	52	7	R	25	7	9									
					-	-	-	-	-	+	+	++	+	0	5	++
30	F	-	-	-	31	5	20									
	-				-	-	-	-	-	-	-	-	-	-	-	-
31	-	-	-	-	38	6	19									
	-				-	-	-	-	-	-	-	-	-	-	-	-

TABLE 1:4

Mean concentrations (ng/ml) of homovanillic acid (HVA), 3,4-dihydroxyphenylacetic acid (DOPAC) and 5-hydroxyindol-3-ylacetic acid (5-HIAA) in the lumbar c.s.f. of patients with parkinsonism and in control patients.

Acids	Parkinsonian	Controls
HVA	19 ± 11 (29)+	53 ± 37 (11)++
DOPAC	6 ± 2 (23)	9 ± 1 (6)
5-HIAA	13 ± 5 (26)	19 ± 4 (21)++

+Mean concentration ± standard deviation (No. of patients).

++Guldberg et al., (1969).

Differences between the two groups are statistically highly significant in respect of all three acids ($p < 0.005$).

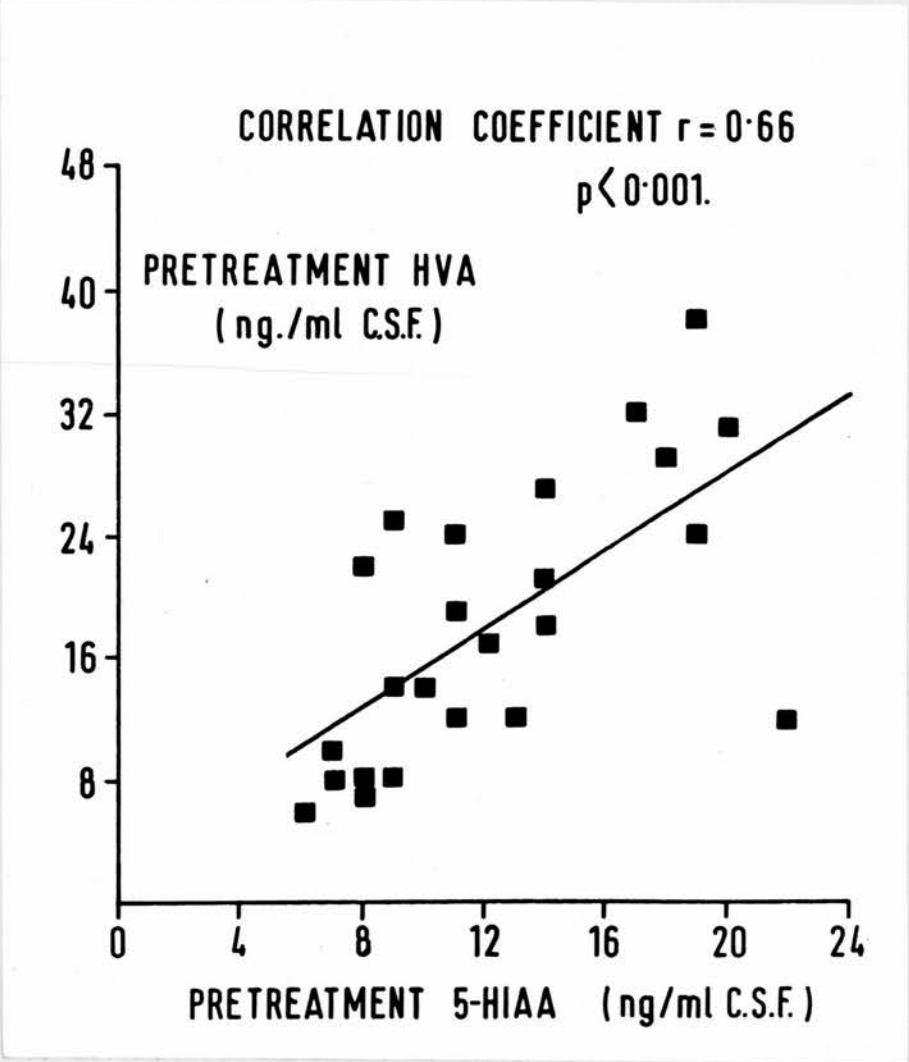


Fig. 1:4

Correlation between the concentrations (ng/ml) of homovanillic acid (HVA) and 5-hydroxyindol-3-ylacetic acid (5-HIAA) in the lumbar c.s.f. of patients with parkinsonism.

the fluorescence monochromator being set at a wavelength of 470 mμ. No DOPA was detectable in the lumbar c.s.f. of patients before they received L-DOPA.

A significant correlation ($p < 0.001$) was observed between the concentrations of HVA and 5-HIAA in the lumbar c.s.f. of patients with parkinsonism (Fig. 1:4). A similar correlation ($r = 0.665$, $p < 0.001$) was obtained by Guldberg (1967) for the concentrations of HVA and 5-HIAA in the ventricular c.s.f. of patients undergoing steriotactic operation for the relief of parkinsonian symptoms.

The patients in the present study were arranged into three approximately equal groups according to age; those up to and including 55 years old; those from 56 - 61 and those over 62 years old. There was found to be no significant difference ($p > 0.05$) between the concentrations of HVA, DOPAC or 5-HIAA in the lumbar c.s.f. of the patients in these three groups (Table 1:5).

A similar grouping was arranged depending on the duration of the illness in the patients; those who had shown symptoms for up to 5 years, from 6 - 9 years and those in whom parkinsonism had been present for more than 10 years. The differences between these groups in the concentrations of the three phenolic acids were not significant ($p > 0.05$) (Table 1:6). Operation for the relief of tremor and rigidity did not significantly alter the concentrations of the three acids (Table 1:7) nor did their concentrations differ significantly between males and females (Table 1:8).

There was also found to be no direct relation between the clinical

TABLE 1:5

Mean concentrations (ng/ml) of homovanillic acid (HVA), 3,4-dihydroxyphenylacetic acid (DOPAC) and 5-hydroxyindol-3-ylacetic acid (5-HIAA) in the lumbar c.s.f. of parkinsonian patients grouped according to age.

Acids	Up to 55 years	56-61 years	over 62 years
HVA	17 ± 7 (9)+	16 ± 10 (8)	17 ± 7 (7)
DOPAC	7 ± 3 (8)	5 ± 3 (6)	7 ± 2 (6)
5-HIAA	11 ± 4 (8)	12 ± 6 (7)	13 ± 4 (6)

+Mean concentration ± standard deviation (No. of patients).
There is no significant difference ($p > 0.05$) between the concentration of any one acid in any of the groups.

TABLE 1:6

Mean concentrations (ng/ml) of homovanillic acid (HVA), 3,4-dihydroxyphenylacetic acid (DOPAC) and 5-hydroxyindol-3-ylacetic acid (5-HIAA) in the lumbar c.s.f. of three groups of patients showing the symptoms of parkinsonism for varying number of years.

Acids	3-5 years	6-9 years	10 years or longer
HVA	16 ± 9 (8)+	18 ± 10 (8)	16 ± 8 (8)
DOPAC	7 ± 4 (6)	6 ± 3 (7)	6 ± 2 (7)
5-HIAA	11 ± 4 (5)	11 ± 5 (8)	13 ± 5 (8)

+Mean concentration ± standard deviation (No. of patients).
There is no significant difference ($p > 0.05$) between the concentration of any one acid in any of the groups.

TABLE 1:7

The mean concentrations (ng/ml) of homovanillic acid (HVA); 3,4-dihydroxyphenylacetic acid (DOPAC) and 5-hydroxyindol-3-ylacetic acid (5-HIAA) in the lumbar c.s.f. of patients having undergone steriotactic operation for the relief of tremor and rigidity and those not having undergone the operation.

Acids	Operated	Non-operated
HVA	16 ± 7 (14)*	16 ± 8 (10)
DOPAC	6 ± 2 (12)	6 ± 3 (8)
5-HIAA	12 ± 4 (13)	12 ± 5 (8)

*Mean concentration ± standard deviation (No. of patients).

There is no significant difference ($p > 0.05$) between the concentrations of any one acid in the two groups.

TABLE 1:8

Mean concentrations (ng/ml) of homovanillic acid (HVA); 3,4-dihydroxyphenylacetic acid (DOPAC) and 5-hydroxyindol-3-ylacetic acid (5-HIAA) in male and female parkinsonian patients.

Acids	Males	Females
HVA	15 ± 7 (16)+	21 ± 9 (9)
DOPAC	6 ± 2 (12)	7 ± 3 (9)
5-HIAA	11 ± 4 (13)	14 ± 4 (10)

+Mean concnetration ± standard deviation (No. of patients).

There is no significant difference ($p > 0.05$) between the concentrations of any one acid in the two groups.

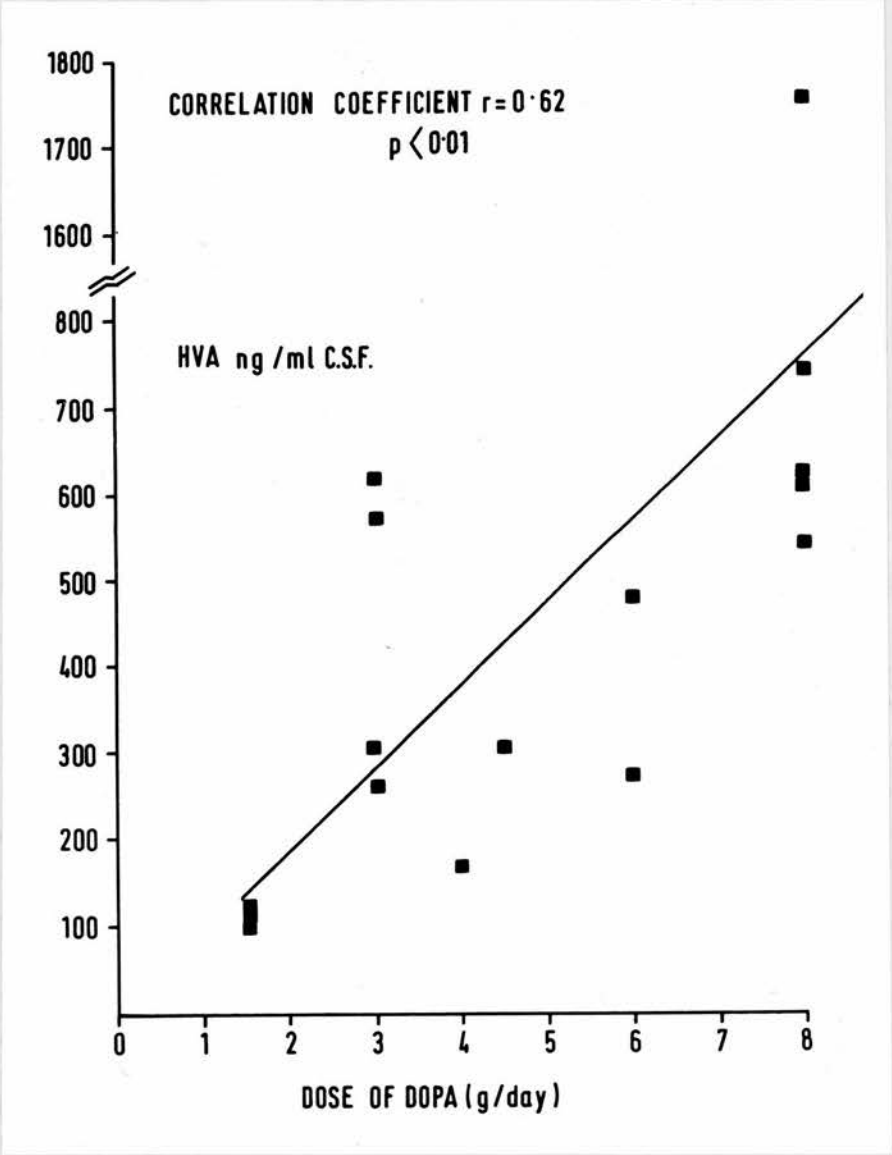


Fig. 1:5

The relation between the concentration (ng/ml) of homovanillic acid (HVA) in the lumbar c.s.f. of parkinsonian patients and the dose (g/day) of L-DOPA.

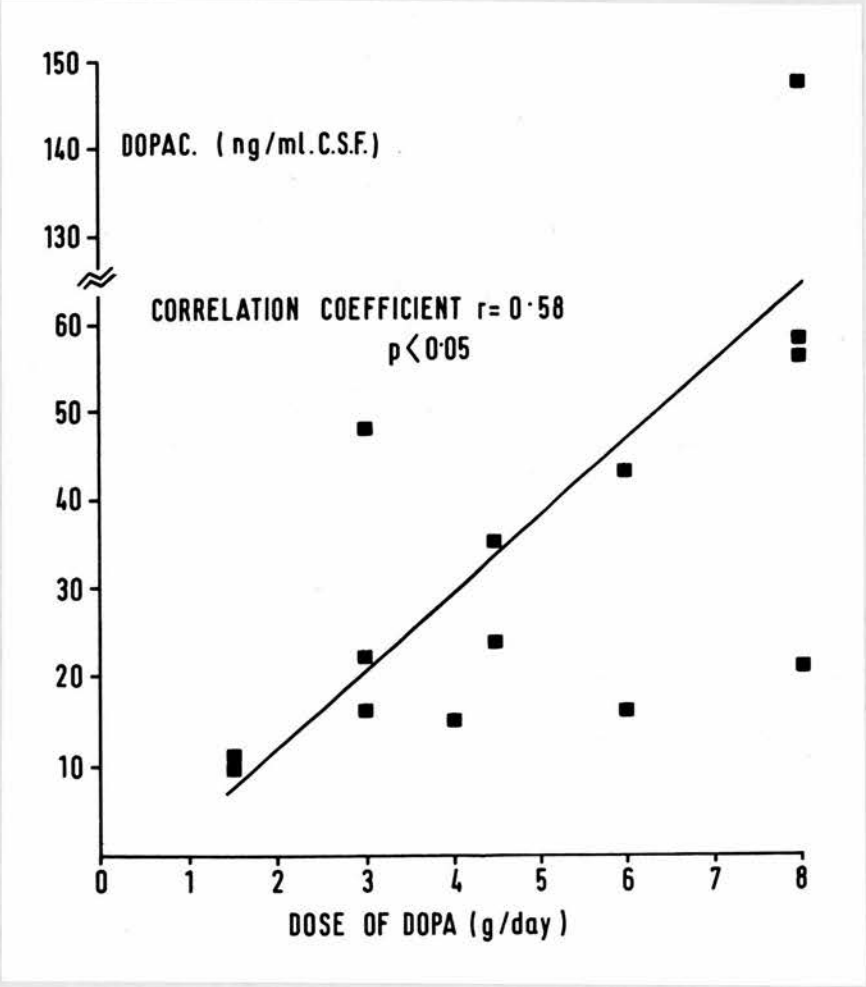


Fig. 1:6

The relation between the concentration (ng/ml) of 3,4dihydroxy-phenylacetic acid (DOPAC) in the lumbar c.s.f. of parkinsonian patients and the dose (g/day) of L-DOPA.

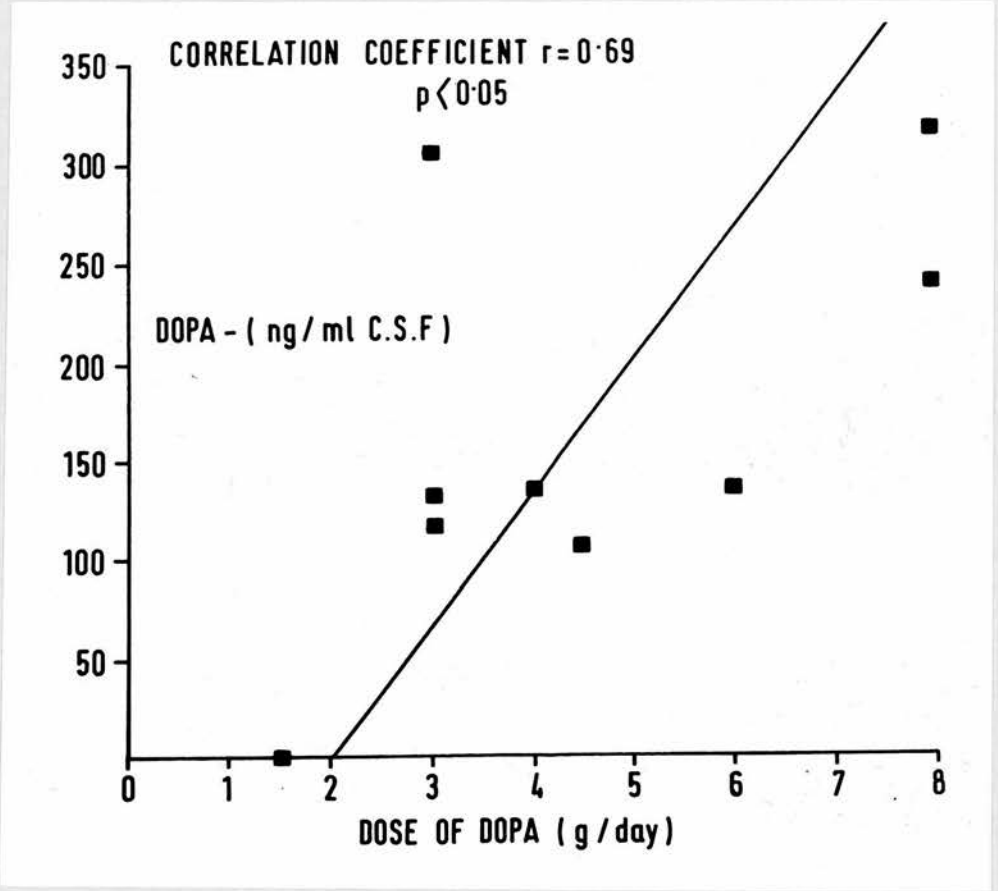


Fig. 1:7

The relation between the concentration (ng/ml) of DOPA in the lumbar c.s.f. of parkinsonian patients and the dose (g/day) of L-DOPA.

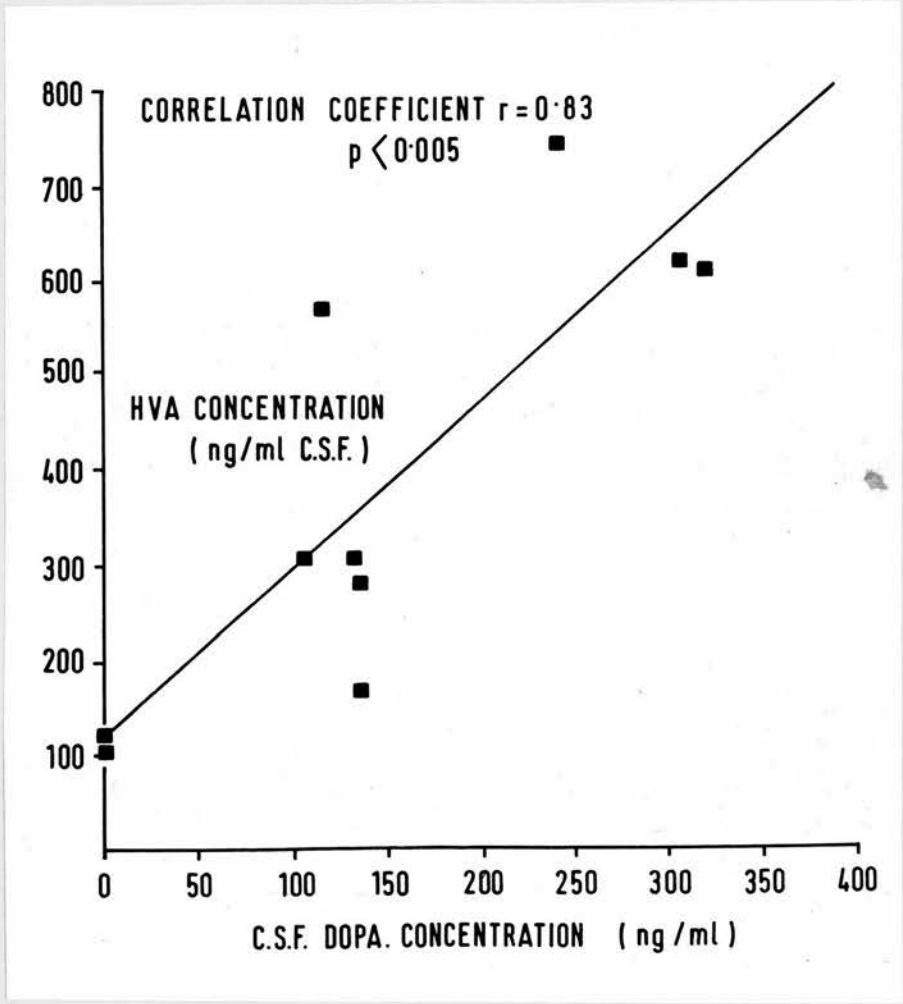


Fig. 1:8

Correlation between the concentrations (ng/ml) of homovanillic acid (HVA) and DOPA in the lumbar c.s.f. of parkinsonian patients treated with different doses of L-DOPA.

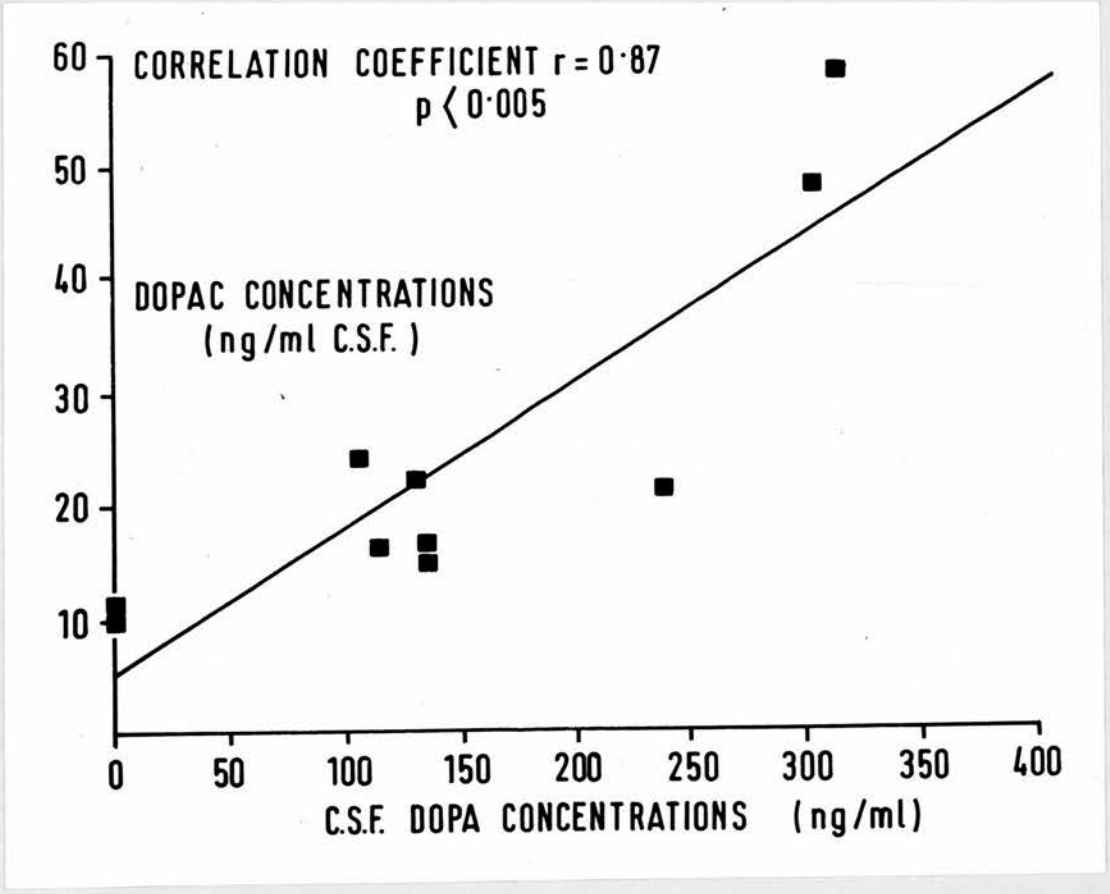


Fig. 1:9

Correlation between the concentrations (ng/ml) of 3,4dihydroxy-phenylacetic acid (DOPAC) and DOPA in the lumbar c.s.f. of parkinsonian patients treated with different doses of L-DOPA.

ratings of these patients and the pre-treatment concentrations of HVA, DOPAC and 5-HIAA in their lumbar c.s.f., nor could the degree of improvement observed on administration of L-DOPA be predicted from their levels.

"On-treatment studies"

The administration of L-DOPA to patients with parkinsonism increased the concentrations of HVA and DOPAC in their lumbar c.s.f. As might be expected, the higher doses of L-DOPA gave rise to higher concentrations of the acids (Fig. 1:5 and 1:6), but the concentrations found in the c.s.f. of patients on any one dose varied considerably. For example, the concentrations of HVA and DOPAC in patients on 8 g of L-DOPA per day ranged from 538 - 1755 ng/ml and 21 - 147 ng/ml respectively.

DOPA was undetectable in lumbar c.s.f. until the dose of the amino acid was greater than 1.5 g/day, above which the concentration increased with increasing dose (Fig. 1:7). The degree of correlation between the concentration of DOPA in the lumbar c.s.f. and the dose of L-DOPA was, however, low and the concentrations of HVA and DOPAC in any one sample showed a greater degree of correlation (r = 0.83 and 0.87 respectively) with the concentration of DOPA in that sample than they did to the dose of L-DOPA (r = 0.62 and 0.58 respectively) administered to the patients at the time of lumbar fluid withdrawal (Fig. 1:8 and 1:9).

The majority of patients in the present study showed slight

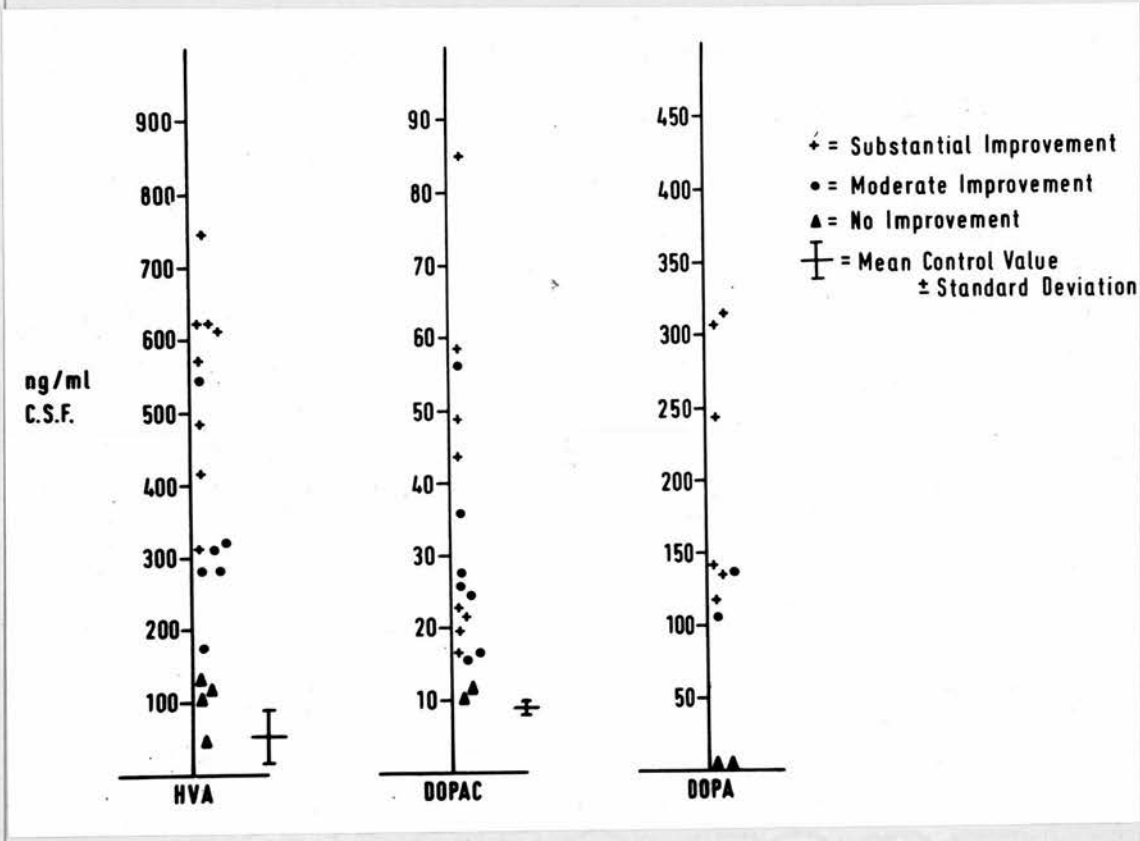


Fig. 1:10

The relation between the concentration (ng/ml) of 3-methoxy-4-hydroxyphenylacetic acid (HVA), 3,4-dihydroxyphenylacetic acid (DOPAC) and DOPA in the lumbar c.s.f. of parkinsonian patients receiving L-DOPA and the clinical improvement observed in these patients.

▲ = No improvement

● = Moderate improvement

\+ = Substantial improvement

increases in the concentration of 5-HIAA in their lumbar c.s.f. on administration of L-DOPA. This increase was independent of the dose received and a paired difference analysis showed that it was not significant ($p > 0.05$).

The features of parkinsonism which showed the greatest improvement were bradykinesis, gait and speech volume (Ahmed, Gillingham, Hanieh, Pullar, Weddell and Ashcroft, 1969). Tremor was variably affected and in most patients it was not altered to any detectable degree, but in certain cases, after several weeks of treatment with L-DOPA it was slightly improved. On the other hand other patients showed an increase in tremor and the aggrevation of this symptom forced discontinuation of the drug. Other involuntary movements such as jerking movements of the arms and legs sometimes developed but these could be reversed or reduced on stopping the drug.

The clinical improvement shown by these patients on being given L-DOPA was associated with increased concentrations of DOPA, DOPAC and HVA in the lumbar c.s.f. as shown in Fig. 1:10. These observations are considered at greater length in the discussion (page 58). Of the 30 patients in the present study about whom the degree of improvement is known, 11 showed substantial improvement, 11 moderate improvement and 8 were not improved by the administration of L-DOPA.

DISCUSSION

The mean concentrations of homovanillic acid (HVA) and 5-hydroxyindol-3-ylacetic acid (5-HIAA) found in the lumbar c.s.f. of patients with parkinsonism before treatment with L-DOPA are similar to those recorded by Johansson and Roos (1967) and Guldberg et al., (1969) (Table 1:9). By a comparison with the control values (Table 1:4, page 35) given by the latter workers, they again demonstrate the lower-than-normal level of these acids as a feature of the syndrome. The present study has extended these observations to include 3,4-dihydroxyphenylacetic acid (DOPAC) which also shows a significantly lower concentration in the c.s.f. of the parkinsonian patients than in the controls (Table 1:4, page 35). This acid has not been measured in the brain of parkinsonian patients, but Sharman, Poirier, Murphy and Sourkes (1967) showed that, in monkeys, lesions in the nigrostriatal tract, which produced parkinsonian-like symptoms, caused a reduction in the concentration of DOPAC in their brains. The finding of lower concentrations of these three acids in the lumbar c.s.f. of parkinsonian patients is consistent with that of Ehringer and Hornykiewicz (1961) and Hornykiewicz (1962) of reduced dopamine levels in the caudate nucleus and substantia nigra in this syndrome and of Bernheimer et al., (1961), of a reduction of 5-hydroxytryptamine in several areas of parkinsonian brain.

The highly significant positive correlation ($r = 0.66$; $p < 0.001$) between the concentrations of HVA and 5-HIAA in the

TABLE 1:9

The concentration (ng/ml) of homovanillic acid (HVA) and 5-hydroxyindol-3-ylacetic acid (5-HIAA) in the lumbar c.s.f. of parkinsonian patients.

	Johansson and Roos (1967)	Guldberg et al. (1969)	Present study
HVA	18 (18)	12 ± 5 (8)+	19 ± 11 (29)
5-HIAA	12 (24)	14 ± 3 (8)	13 ± 5 (26)

+Mean concentration ± standard deviation (No. of patients).

lumbar c.s.f. of parkinsonian patients prior to the administration of L-DOPA is not fully understood. Guldberg (1967) observed a similar correlation ($r = 0.665$; $p < 0.001$) between the concentration of these two acids in the ventricular c.s.f. of parkinsonian patients but showed that no such correlation existed in the control patients. These findings may reflect the homogeneity of the group of parkinsonian patients and the heterogeneity of the control group.

In parkinsonism the severity of symptoms becomes greater the longer a person has had the disease. There was, however, no significant correlation between the concentration of HVA, DOPAC or 5-HIAA in the lumbar c.s.f. of the patients and the length of time they had had the illness (Table 1:6, page 39). This is in contrast to the findings of Guldberg et al., (1967) that the concentrations of HVA and 5-HIAA in the ventricular c.s.f. were lower in patients who had shown parkinsonian symptoms for ten years or longer, than they were in patients who had had the disease for a shorter time. This difference between the findings in the lumbar c.s.f. in the present study and the ventricular c.s.f. by Guldberg et al., (1967) probably arises from the close proximity of the ventricular c.s.f. to the caudate nucleus. This area of brain, along with the putamen, shows the greatest alteration in dopamine and 5-hydroxytryptamine content in parkinsonism (Hornykiewicz, 1962) and small quantitative alterations in the metabolism of these two amines are more likely to give rise to detectable changes in the concentration of HVA and 5-HIAA in the

ventricular c.s.f. where the levels of these acids are high, than in the more remote lumbar c.s.f. which has much lower concentrations of HVA and 5-HIAA.

Although parkinsonism is progressive, the rate of deterioration varies between individuals, and patients who have had the disease for the same length of time may show varying severity of symptoms. There was, however, no indication of a relation between the severity of the symptoms shown by these patients and the concentration of any of the phenolic acids in the c.s.f.. There was also no evidence of dependence of the concentration of HVA, DOPAC or 5-HIAA on the age (Table 1:5, page 38) or sex (Table 1:8, page 41) of the patients nor was any significant difference observed between the concentrations in the lumbar c.s.f. of patients who had undergone unilateral or bilateral steriotactic surgery for the relief of tremor and rigidity and those who had not (Table 1:7 page 40)

The reason for the low dopamine and 5-hydroxytryptamine concentrations in the brains of parkinsonian patients (Hornykiewicz, 1962) is not fully understood. There may be a diminished capacity for storage of these amines. This is supported by the fact that reserpine, which is known to release brain amines from their stores (Güldberg and Yates, 1968) can produce parkinsonian-like symptoms when administered to man in high doses (May and Voegele, 1956). However, if normal rates of synthesis persist, this diminished capacity for storage would not reduce the concentrations of the acidic catabolites of these amines

in the c.s.f.

A diminished ability of the brain to synthesise these amines could also be responsible for their lower concentration in the brain of parkinsonian patients. However it has been reported (Bernheimer and Hornykiewicz, 1962) that the activity of aromatic amino acid decarboxylase, which catalyses the decarboxylation of L-DOPA to dopamine and of 5-hydroxytryptophan to 5-hydroxytryptamine is not reduced in the brains obtained post mortem from parkinsonian patients. Aromatic amino acid decarboxylase is abundant in the cerebral capillary walls (Bertler, Falck and Rosengren, 1963; Constantinidis, Bartholini, Tissot and Pletscher, 1968; Constantinidis, de la Torre, Tissot and Geissbuhler, 1969) and there could thus conceivably be a large change in the intraneuronal activity of this enzyme without a detectable change in the total.

A second enzyme involved in the synthesis of dopamine is tyrosine hydroxylase. This enzyme converts tyrosine to DOPA and is the rate-limiting step in the formation of the catecholamines (Nagatsu, Levitt and Udenfriend, 1964). A reduction in the activity of this enzyme in the brains of parkinsonian patients would lead to a diminished synthesis of dopamine. It has been reported (Goldstein, Anagnoste, Owen and Battista, 1966) that in monkeys with parkinsonian-like symptoms caused by ventromedial tegmental lesions, there is a reduction in the activity of tyrosine hydroxylase in the striatum on the same side as the lesion. These authors found that there was a 90% decrease in the ability of the striatum ipsilateral

to the lesion to synthesise substances with a catechol grouping from intraventricularly administered ^{14}C-tyrosine and that an homogenate of the caudate nucleus and putamen on this side had a 50% lower capacity to convert ^{14}C-tyrosine to catechol-containing compounds than the same areas contralateral to the lesion.

As a result of the lowered levels of dopamine in the basal ganglia in parkinsonian patients, there may exist in this condition an imbalance between the activity of dopaminergic and cholinergic systems in the brain (Barbeau, 1962). This suggestion is supported by the finding that parkinsonian-like symptoms can be produced by drugs such as reserpine and chlorpromazine (May and Voegele, 1956) which are known to affect the activity, release or metabolism of dopamine (Laverty and Sharman, 1965; Guldberg and Yates, 1968) and by substances such as physostigmine which facilitate cholinergic transmission (Duvoisin, 1967). Further, circumstantial evidence is provided by a consideration of the methods available for treatment of the syndrome, suppression of the cholinergic system being achieved with anticholinergic, antiparkinsonian agents and probably by surgery.

In an attempt to raise the brain dopamine concentrations and thus the functional level of the dopaminergic neurones, several workers (Birkmayer and Hornykiewicz, 1961; Barbeau, 1962; M^{c}Geer and Zeldowicz, 1964; Cotzias et al., 1969; Calne, Sterne, Laurence, Sharkey and Armitage, 1969b; Curzon, Godwin-Austen, Tomlinson and Kantamaneni, 1970) have given the dopamine precursor, DOPA, to patients with parkinsonism. The amino acid, DOPA, rather than

dopamine itself was used as dopamine will not pass the blood brain barrier (Pletscher and Gey, 1962) and in most cases L-DOPA was administered to the patients as D-DOPA is not taken into brain *in vivo* (Gey and Pletscher, 1964). Tyrosine, another amino acid precursor of dopamine has not been used, as the conversion of tyrosine to DOPA, catalysed by tyrosine hydroxylase, is the rate-limiting step in the formation of the catecholamines (Nagatsu *et al.*, 1964) and is probably already functioning at maximum capacity (Calne and Sandler, 1970).

The absorption of DOPA from the gut and its subsequent metabolism when administered orally to parkinsonian patients was shown by M^{c}Geer and Zeldowicz (1964), who obtained an increase in the dopamine concentration in the urine of these patients of between 100 and 1,000 times after the oral administration of up to 5 g of D,L-DOPA. These findings were confirmed by Smith (1967) who obtained an increase in the concentration of the dopamine metabolite, HVA, in the urine of normal human subjects after giving them an oral dose of L-DOPA. Calne *et al.*, (1969a) also found an increase in the urinary concentration of HVA as well as of DOPAC and other dopamine metabolites, after giving parkinsonian patients 0.5 - 2.5 g of L-DOPA per day. As these authors point out, however, the alteration in the concentration of the urinary metabolites of DOPA is probably a reflection of its peripheral metabolism and not of its metabolism within the central nervous system.

Because of difficulties in obtaining samples, it has not been

possible to show an increase in the concentration of dopamine in human brain after the administration of DOPA. In rats, however it has been reported (Gey and Pletscher, 1964), that the administration of D,L-(^{14}C)-DOPA gives rise to a ^{14}C-labelled amine as well as an acid fraction in the brain and Poirier, Singh, Sourkes and Boucher (1967) have shown that in cats the intraperitoneal injection of DOPA increased the dopamine concentration in the striata. Indirect evidence for the absorption into and metabolism of L-DOPA in the central nervous system of man was obtained by Pletscher, Bartholini and Tissot (1967) who found ^{14}C-HVA and ^{14}C-3-O-methylDOPA in the lumbar c.s.f. of normal human subjects given intravenous doses of L-(^{14}C)-DOPA. Further indirect evidence for the cerebral metabolism of peripherally administered L-DOPA was reported by Curzon et al., (1970) who obtained increases in the HVA concentration in the lumbar c.s.f. of parkinsonian patients treated with up to 7 g of L-DOPA per day. That the presence of HVA in the lumbar c.s.f. is not due to the transfer from the blood of HVA formed in the periphery has been shown by Pletscher et al., (1967), who obtained no significant labelling of the HVA in the lumbar c.s.f. of normal humans after the intravenous injection of ^{14}C-HVA. The lack of penetration of HVA from blood to c.s.f. has also been demonstrated in the dog by Guldberg and Yates (1968). These authors reported that the intravenous infusion of HVA over a period of 1 hour did not significantly increase the concentration of HVA in the lateral ventricular c.s.f. even though the concentration of HVA in the

plasma was raised above that in the c.s.f.

Evidence that an increased cerebral synthesis of dopamine has resulted from L-DOPA administration and the possible relationship of such an increase to clinical improvement in parkinsonian patients has been sought in the present investigation. On administration of L-DOPA the concentration of HVA and DOPAC in the lumbar c.s.f. increased. The rise in HVA concentration was substantially greater than that of DOPAC, contrasting with the findings of Calne et al (1969a) of an increase in urinary DOPAC concentrations, after L-DOPA administration, equal to or greater than the increase in HVA. The significance level ($p < 0.05$) of the correlation between the concentration of HVA in the lumbar c.s.f. and the dose of L-DOPA was the same as that obtained by Curzon et al., (1970).

Attempts to relate the c.s.f. levels of HVA and DOPAC to the clinical improvement must at best remain superficial since clinical considerations preclude manipulation of the experimental situation such as maintenance on subeffective dosage and multiple c.s.f. sampling, which would be necessary to complete the correlation profiles. However, within the limitations imposed in the present study, the following observations may be made. Two patients receiving 1.5 g L-DOPA per day failed to show any clinical improvement in spite of the fact that the DOPAC and HVA concentrations in the lumbar c.s.f. had risen to within the normal physiological range; 11 ng and 10 ng/ml for DOPAC and 103 and 116 ng/ml for HVA. At this dose level, however, no DOPA was detectable in the c.s.f.

The remaining patients on doses of 3 g or more of L-DOPA per day showed clinical improvement associated with c.s.f. DOPAC levels in excess of 15 ng/ml and c.s.f. HVA levels in excess of 250 ng/ml. Both these values deviate from the mean control levels (Table 1:4) by six standard deviations. In excess of these critical values the degree of clinical improvement did not relate to the levels of the two acids in the c.s.f. (Fig. 1:10, page 48).

In explanation of the observation that the c.s.f. concentrations of DOPAC and HVA require to reach higher than normal levels before obvious improvement in the clinical status becomes apparent, it is probable that only a very small proportion of these metabolites arises from the turnover of cerebral dopamine derived from the administered L-DOPA. By means of fluorescence microscopy (Falck, Hillarp, Thieme and Thorp, 1962; Hamberger, Malmfors and Sachs, 1965) and biochemical analysis, Constantinidis, Bartholini, Tissot and Pletscher (1968) and Constantinidis, de la Torre, Tissot and Geissbuhler (1969) have demonstrated the conversion of L-DOPA to dopamine and the subsequent metabolism of the dopamine within the endothelial cells of the cerebral capillaries of rats. The peripheral administration of L-DOPA to these animals increased the fluorescence in the cerebral capillary endothelium without causing any increase in the fluorescence of the parenchyma of the brain. It is not possible by means of fluorescence microscopy to distinguish between DOPA and dopamine but these authors were able to show that the observed increase in fluorescence was, at least in

part, due to dopamine by using a specific biochemical anaylsis which indicated an increase in the concentration of this amine in the pallidum and striatum. The dopamine in the endothelium of the capillaries was formed within these cells and did not enter from the blood as the peripheral administration of dopamine produced a fluorescence only in the lumen, and not in the endothelium, of the capillaries. The DOPA-induced fluorescence in the capillary endothelium and the dopamine concentration in the pallidum and striatum were markedly elevated by pre-treatment of the rats with the monoamine oxidase inhibitor nialamide. This finding indicated that the dopamine formed within the endothelial cells of the cerebral capillaries was also being metabolised there by monoamine oxidase to give, presumably, HVA and DOPAC which may from there be capable of entering the c.s.f.

In addition to different cellular sites of formation of dopamine and its metabolites, there is the possibility of different metabolic routes of formation of the acids. For example, HVA could, theoretically, be derived from L-DOPA through oxidative deamination of 3-O-methyldopamine formed by decarboxylation of 3-O-methylDOPA. This pathway would bypass the formation of the biologically active amine, dopamine, in the production of HVA. The O-methylated amino acid and HVA were the only labelled metabolites found by Pletscher et al., (1967) in the c.s.f. of human subjects given L-(^{14}C)-DOPA intravenously. However, DOPA decarboxylase has been reported to be less than 10% as active towards 3-O-methylDOPA as it is towards

L-DOPA (Ferrini and Glasser, 1964). Whilst the extensive formation of 3-O-methylDOPA seems, on present evidence, unlikely, under conditions of DOPA load the concentration of 3-O-methylDOPA may be such that its metabolism via DOPA decarboxylase is a significant factor in the production of HVA.

The amount of DOPA entering the central nervous system of patients receiving the same oral dose of L-DOPA will vary with differences in absorption from the gut, peripheral metabolism and in penetration into the central nervous system. Such factors probably account for the wide range of the concentrations of DOPA and its metabolites observed in the c.s.f. of patients on the same oral dose of L-DOPA and also for the better degree of correlation of the post-treatment HVA and DOPAC concentrations with the lumbar c.s.f. DOPA levels (r =0.83 and 0.87 respectively) than with the dose of L-DOPA received by the patients at the time of lumbar fluid withdrawal (r = 0.62 and 0.58 respectively). In rats, a barrier to the entry of L-DOPA from the blood stream into the brain tissue has been shown to exist (Constantinidis et al., 1968; Constantinidis et al., 1969). This barrier was enzymatic in nature and was due to the metabolism of L-DOPA to dopamine by means of the DOPA decarboxylase within the endothelial cells of the cerebral capillaries. If a similar barrier exists in the brains of humans L-DOPA will not enter the brain tissue in any significant amounts until its concentration in the blood is such that the DOPA decarboxylase within the capillary endothelium is saturated. Constantinidis et al., (1968) and Constantinidis et al.,

(1969) were able to overcome this barrier by pre-treatment of the rats with the DOPA decarboxylase inhibitor N^1-(D,L-seryl)-N^2-(2,3,4-trihydroxybenzyl)-hydrazine hydrochloride (Ro 4-4602) and thus allow the entry of the L-DOPA into the brain parenchyma where it was metabolised to dopamine. Ro 4-4602, when given peripherally to rats, does not enter the brain and thus only inhibits the DOPA decarboxylase in the periphery (Bartholini, Bates, Burkard and Pletscher, 1967; Bartholini and Pletscher, 1968). In order to increase the proportion of any one dose of L-DOPA entering the central nervous system, Bartholini, Tissot and Pletscher (1969) treated parkinsonian patients with L-DOPA together with Ro 4-4602 and obtained significant improvement with low oral doses (450 - 600 mg) of L-DOPA. These doses of L-DOPA are very much lower than those of between 3 and 8 g which were required in order to obtain significant improvement in the parkinsonian patients in the present study.

The treatment of parkinsonian patients with L-DOPA seems to have no effect on the metabolism of 5-hydroxytryptamine, as administration of L-DOPA in doses up to 8 g per day did not significantly alter the concentrations of the 5-hydroxytryptamine metabolite, 5-HIAA, in the lumbar c.s.f. That an interaction between the metabolic pathways of the catecholamines and the 5-hydroxyindolalkylamines can occur was demonstrated by Brodie, Comer, Costa and Dlabac (1966) who found that the intravenous injection of 500 mg/kg of a 5-hydroxytryptamine precursor, 5-hydroxytryptophan, into rats reduced the noradrenaline levels in their brains by 50%. A similar reduction

in the noradrenaline levels in the brain stem of rabbits was obtained by these authors on the intravenous injection of 200 mg/kg of 5-hydroxytryptophan. An interaction between the metabolic pathways of 5-hydroxytryptamine and dopamine was also postulated by Moir (1969) who found that, in dogs, the intravenous injection of 50 mg/kg of the primary 5-hydroxytryptamine precursor, L-tryptophan, followed by the intravenous infusion of 20 mg/kg/hr of this amino acid, increased the concentration of the dopamine metabolite, HVA, in the cisternal c.s.f. to a value 14 times that of the control value, with a concomitant threefold rise in the concentration of the 5-hydroxytryptamine metabolite, 5-HIAA. Conversely, Guldberg and Yates (1968) have demonstrated that in dogs the intravenous injection of 25 mg/kg of the dopamine precursor, L-DOPA, does not alter the concentration of 5-HIAA in the lateral ventricular c.s.f. as measured over a period of 4 hours. It thus seems that where as an increase in the concentration of 5-hydroxytryptamine in the brain, caused by the administration of one of its amino acid precursors, can dislodge dopamine or noradrenaline from its stores, an increase in the concentration of dopamine in the brain, produced by the administration of L-DOPA, has no such effect on 5-hydroxytryptamine.

The administration of L-DOPA to parkinsonian patients relieves, in the main, akinetic symptoms and causes only marginal improvement, and may even aggravate the tremor and rigidity. Steriotactic operation or the use of anticholinergic drugs on the

other hand have their major effect on tremor and rigidity and do not alter the akinetic state of the patient. The theory of an imbalance of a dopaminergic and cholinergic pathway (Barbeau, 1962) and the stimulation of the dopaminergic pathway by the administration of L-DOPA and the inhibition of the cholinergic pathway by operation or by the use of anticholinergic antiparkinsonian drugs, is not sufficient to explain this discrepancy. As L-DOPA is a precursor of dopamine it is also a precursor of noradrenaline (Levin, Levenberg and Kaufman, 1960) another biogenic amine which is present in reduced concentrations in the brain of parkinsonian patients (Ehringer and Hornykiewicz, 1960; Hornykiewicz, 1962). The administration of this amino acid to the parkinsonian patients may thus cause normalisation of the functional state of noradrenergic nervous pathways in the brain.

On the oral administration of L-DOPA to parkinsonian patients, Calne et al., (1969a) found that in the urine the concentration of the noradrenaline metabolite, 3-methoxy-4-hydroxy-mandelic acid (VMA) was only very slightly increased whilst the concentration of another noradrenaline metabolite, 3-methoxy-4-hydroxyphenylethyleneglycol, was not significantly altered. Urine levels of the catecholamines and their metabolites, however, are not necessarily a good index of cerebral metabolism. This is shown by the concentration of noradrenaline in the urine of parkinsonian patients which falls within the normal range (Barbeau et al., 1961) whilst its concentration in the brain of these patients is reduced by up to 50%

(Hornykiewicz, 1962). There may, therefore, be a significant change in the concentration of noradrenaline in the brain of parkinsonian patients given L-DOPA with only minimal, if indeed any, change in the urinary excretion of this amine or its metabolites. There thus exists a need to measure the metabolites of noradrenaline in the c.s.f. of parkinsonian patients both before and after the administration of L-DOPA and to compare these levels with those in the c.s.f. of a control group. The main cerebral metabolite of noradrenaline is 3-methoxy-4-hydroxyphenylethyleneglycol (Rutledge and Jonason 1967; Schanberg, Schildkraut, Breese and Kopin, 1968) and no satisfactory routine method for its estimation has been described in the literature. In the next section of this thesis is described the development of such a method.

The study of the metabolite of the biogenic amines in the lumbar c.s.f. of the human is, under normal circumstances, the closest approach which can be made to a determination of any abnormality in the metabolism of these amines in the central nervous system in various pathological conditions. That the concentration of HVA in the lateral ventricular c.s.f. of parkinsonian patients and control patients can be an index of the change in the concentration of HVA in the brain was postulated by Guldberg et al., (1969) who obtained a ratio of 7.5 for the concentration of HVA in the caudate nucleus to that in the lateral ventricular c.s.f. of the control patients and a similar ratio of 6.4 for the parkinsonian patients, even though the concentration of this acid in the lateral ventricular

c.s.f. of the latter group was reduced by more than 50%. These ratios agree well with that of 6.3 for the dog, an animal which has an HVA concentration in its caudate nucleus about 4 times that in man (Guldberg et al., 1969). The close correlation between the concentration of HVA in the lateral ventricular c.s.f. and that in the caudate nuclei is probably due to the near proximity to the lateral ventricles of these nuclei, areas of brain with an especially high concentration of dopamine. The lumbar c.s.f. is more remote from the caudate nucleus but there is still a constant ratio of 62.5 for parkinsonian patients and 63.7 for control patients for the concentration of HVA in the caudate nucleus compared with that in the lumbar c.s.f. (Table 1:10). Under conditions of L-DOPA load, however, the high concentrations of HVA in the lumbar c.s.f. of parkinsonian patients is probably no longer a good index of the situation in the caudate nucleus, because, as has been pointed out previously, there is more than one possible site of metabolism of L-DOPA capable of giving rise to HVA in the c.s.f. The relationship between the concentration of HVA and DOPAC in the lumbar c.s.f. and the turnover of cerebral dopamine, after L-DOPA administration is, therefore, unknown and it is also unknown whether the therapeutic action of the amino acid is directly due to an increase in the concentration of dopamine in the brain. There is a possibility that its therapeutic action is due to an increase in the concentration of a metabolite of L-DOPA other than dopamine. Within these limitations, studies on the c.s.f. are useful in giving further insight into the mechanism of the disease and its treatment.

TABLE 1:10

Mean concentration of homovanillic acid (HVA) in the caudate nucleus and lumbar c.s.f. of parkinsonian and control patients.

	Caudate nucleus concentration µg/g tissue (C.N.)	lumbar c.s.f. conc. µg/ml c.s.f. (C.S.F.)	Ratio CN/C.S.F.
Control	3.38*	0.053++	63.7
Parkinsonian	1.19+	0.019	62.5

*Bernheimer and Hornykiewicz (1965).

+Bernheimer, Birkmayer, Hornykiewicz, Jellinger and Seitelberger (1966).

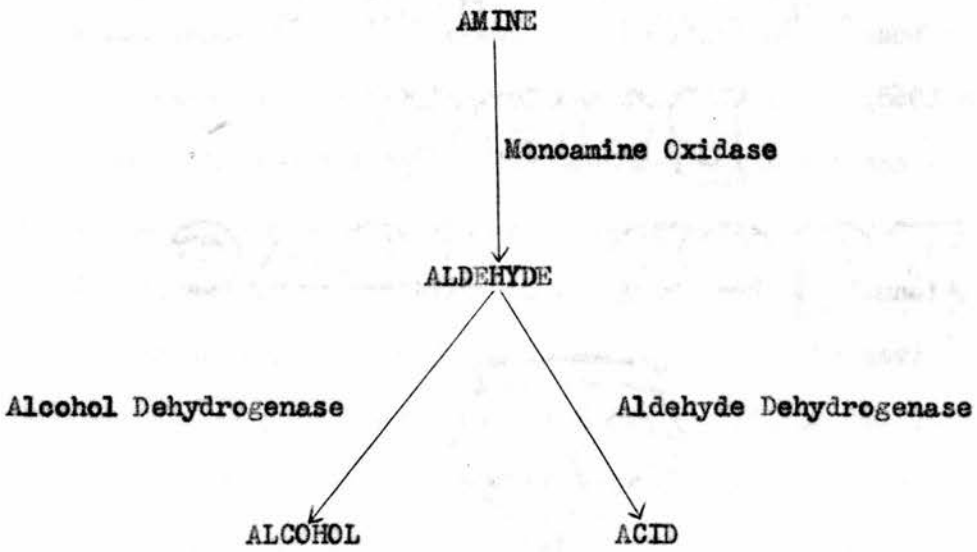
++Guldberg, Ashcroft, Turner and Hanieh (1969).

SECTION 2.

The development of a fluorimetric method for the estimation of 3,4-dihydroxyphenylethyleneglycol and 3-methoxy-4-hydroxyphenylethyleneglycol in biological material.

Fig. 2:1

Metabolic pathway of the catecholamines.



INTRODUCTION

The catabolism of the catecholamines, dopamine and noradrenaline by monoamine oxidase gives rise to compounds containing an aldehyde group which, theoretically, can be either reduced to alcohols by the action of alcohol dehydrogenase or oxidized to acids by aldehyde dehydrogenase (Fig. 2:1). Recently it has been shown (Rutledge and Jonason,1967; Breese, Chase and Kopin,1968; Breese, Chase and Kopin,1969) that phenylethylamines such as noradrenaline and octopamine, which contain a β -hydroxyl group are metabolised by cerebral tissue, both <u>in vivo</u> and <u>in vitro</u>, predominantly to neutral glycols, while the major deaminated catabolites of the metabolism of phenylethylamines which are not hydroxylated in the β - position are acids.

These findings are consistent with previous observations. For instance, the major cerebral metabolite of dopamine is 3-methoxy-4-hydroxyphenylacetic acid (homovanillic acid, HVA) which was first shown to be present in brain tissue by Andén, Roos and Werdinius (1963a) and Sharman (1963) and in cerebrospinal fluid (c.s.f.) by Andén, Roos and Werdinius (1963b). Another acid metabolite of dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC) has also been shown to be present in brain (Rosengren 1960) and c.s.f. (Ashcroft, Crawford, Dow and Guldberg,1968) in concentrations about one tenth of that of HVA (Andén, Roos and Werdinius,1964;

Ashcroft et al., 1968). The presence of endogenous neutral phenylethanol metabolites of dopamine has, as yet, not been demonstrated in the brain or c.s.f. Goldstein, Friedhoff, Pomerantz and Simmons (1960), however, have shown that ^{14}C-3-methoxy-4-hydroxyphenylethanol (MOPET) was present in the urine of rats treated with ^{14}C-dopamine. The catechol-containing ethanol, 3,4-dihydroxyphenylethanol (DOPET) was not detectable in the urine unless the rats were pretreated with the catechol-O-methyl transferase inhibitor, pyrogallol (Goldstein, Friedhoff, Pomerantz and Contrera, 1961). These authors also showed that homogenates of rat brain were capable of converting dopamine to DOPET, no MOPET was formed as homogenates of brain tissue will not carry out the O-methylation reaction (Goldstein, Friedhoff and Simmons, 1959).

The major acid metabolite of noradrenaline in the urine, 3-methoxy-4-hydroxymandelic acid (vanillin mandelic acid, VMA) (Pisano, Crout and Abraham, 1962; Georges, 1964) has not been detected in brain tissue (Laverty and Sharman, 1965). Another acid metabolite of noradrenaline, 3,4-dihydroxymandelic acid (DOMA) which has been shown to be present in urine (Weise, McDonald and La Brosse, 1961) has been reported to be present in the brains of rabbits (Matsuoka, Yoshida and Imaizumi, 1964). This report, however, has never been substantiated. A neutral metabolite of noradrenaline, 3-methoxy-4-hydroxyphenylethyleneglycol (MOPEG) was first identified in the urine of rats and of man after the peripheral administration of labelled noradrenaline (Axelrod,

Kopin and Mann, 1959). This glycol was excreted in the urine as its sulphate ester and accounted for 35% of the administered dose of noradrenaline in the rat but only 5% in man. These findings were confirmed for the rat by Kopin, Axelrod and Gordon, (1961) and for man by La Brosse, Axelrod, Kopin and Kety (1961). Kirshner, Terry and Pollard (1961) found MOPEG as its sulphate ester in the urine of cats given an intravenous infusion of D,L-adrenaline-2-^{14}C. These authors also found 3,4-dihydroxy-phenylethyleneglycol (DOPEG) in the urine after pretreatment of the cats with the catechol-O-methyl transferase inhibitor, pyrogallol. MOPEG, as its sulphate ester, has also been shown to be a normal constituent of human urine and to be present in concentrations approximately half that of VMA (Ruthven and Sandler, 1965; Wilk, Gitlow, Clarke and Paley, 1967; Calne, Karoum, Ruthven and Sandler, 1969a). Of this MOPEG excreted in the urine, 22-27% has had its origin in the brain pool of noradrenaline but only 0.7-1.0% of the VMA has the same origin (Maas and Landis, 1968). The ability of cerebral tissue to convert noradrenaline to MOPEG and DOPEG was shown for the cat by Mannari*o, Kirshner and Nashold, (1963). Glowinski, Kopin and Axelrod (1965) found that the major deaminated metabolite formed in the brain of rats from intraventricularly administered noradrenaline was MOPEG, and Schanberg, Schildkraut, Breese and Kopin (1968) demonstrated that the sulphate conjugate of MOPEG was the major metabolite of normetanephrine-^{3}H injected into the cisterna magna of rats.

These authors showed that the MOPEG isolated by them was indeed conjugated with sulphate as it only produced a compound with the same chromatographic properties as authentic MOPEG when it was treated with a mixed sulphatase and glucuronidase preparation and not when it was treated with a pure glucuronidase or phosphatase preparation.

Recently, Schanberg, Breese, Schildkraut, Gordon and Kopin (1968) have estimated the endogenous concentration of both the free and conjugated MOPEG in the brains of several animals and also in human c.s.f. The ratio of free to conjugated MOPEG varied from animal to animal, the rat and guinea pig having no free glycol while the cat and the rhesus monkey had very little in the conjugated form. The green monkey had both free and conjugated MOPEG present in its brain, the concentration of the conjugated form being two to three times that of the free form. In human c.s.f. MOPEG was present mostly in the conjugated form. The concentration in the c.s.f. from the cerebral ventricles (250-400ng/ml) was greater than that in the lumbar c.s.f. (50-200 ng/ml). The concentrations of free MOPEG as well as of DOPEG have also been estimated in a specific area, the hypothalamus, of the brain of the rabbit, mouse and cat (Sharman, 1969). This author in addition to confirming the presence of free MOPEG in the brain has shown that free DOPEG is also present.

Methods used for the identification and estimation of the neutral and acidic metabolites of noradrenaline.

Colorimetric and U.V. absorption. When VMA or MOPEG are oxidised in alkaline solution by periodate they form 3-methoxy-4-hydroxybenzaldehyde (vanillin). This reaction forms the basis for the method used by Pisano et al. (1962) for the estimation of VMA and by Ruthven and Sandler (1965) for MOPEG in human urine. The vanillin was extracted into toluene and returned to an aqueous phase by shaking with a potassium carbonate solution. The optical density of the alkaline solution of vanillin was determined at 360 mμ. Georges (1964) modified this method by coupling the aldehyde group of the vanillin with 2,4-dinitrophenylhydrazine to form a condensation product which adsorbed light with a wavelength of 480 mμ. This method was less specific than that used by Pisano et al. (1962) and Ruthven and Sandler (1965) as the 2,4-dinitrophenylhydrazine condensed with other aldehydes to give compounds which also had a strong absorption at 480 mμ.

Radioactive labelling. The metabolism of noradrenaline has been studied mainly using radioactively labelled compounds (Axelrod et al. 1959; Kirshner et al. 1961; Kopin et al. 1961; La Broose et al. 1961; Mannario et al. 1963; Glowinski et al. 1965; Maas and Landis, 1968; Schanberg, Schildkraut, Breese, and Kopin, 1968). The use of such

compounds to study metabolic pathways depends on an ability to separate and identify the particular metabolites as the methods of estimation are only specific for the label atom and not for the labelled compound. Identification is normally achieved by comparison of the chromatographic mobility of the metabolite in several solvent systems with that of the authentic compound. The sensitivity of detection of the metabolites, which can be in the picogram range, depends on the specific activity of the original substance administered to the animal.

Gas chromatography. Gas chromatography was used by Wilk et al. (1967) for the estimation of MOPEG in human urine. These authors converted the glycol to its trifluoracetate ester and used the very sensitive electron capture technique for its detection. This method was also used by Schanberg, Breese, Schildkraut, Gordon and Kopin, (1968) for the estimation of MOPEG in the brains of various animals and also in human c.s.f.. Electron capture detection is especially sensitive to the presence of halogen atoms and the greater the number of these that can be attached to the substance the greater the sensitivity of its detection. Sharman (1969) estimated MOPEG and DOPEG by forming their heptafluorobutyrate esters; two heptafluorobutyrate residues combining with one of the glycol. The sensitivity of this method was up to fifty times greater than that of Wilk et al. (1967)

Flame ionisation detection has also been used for the estimation of MOPEG and VMA as well as a number of other metabolites of dopamine and noradrenaline present in the urine of parkinsonian patients both before and during treatment with L-DOPA (Calne et al., 1969a). These authors converted the acidic metabolites to their methyl esters / trimethylsilyl ethers and the neutral alcoholic metab metabolites to their trimethylsilyl ether / esters.

Fluorimetry. Fluorimetric analysis has been used by many workers for the estimation of the phenolic acid metabolites of dopamine. These methods are relatively sensitive, allowing estimation in the nanogram range.

There has been no extensive use of fluorimetric techniques for the estimation of the metabolites of noradrenaline. The method of Rosengren (1960) for the estimation of DOPAC can be used for the estimation of DOMA and DOPEG (see page 98). Goldenberg and White (1962) describe briefly a method for the fluorimetric estimation of VMA. This consists of condensing vanillin, formed from the VMA (Pisano et al., 1962) with thiosemicarbazide to give a fluorescent thiosemicarbazone. There is no description in the literature of the estimation of MOPEG by fluorimetry.

The object of the work described in this section of the thesis was to develop fluorimetric methods which could be used routinely for the estimation of MOPEG and DOPEG in human lumbar c.s.f..

METHODS

THE FLUORIMETRIC ESTIMATION OF 3-METHOXY-4-HYDROXYPHENYLETHYLENE-GLYCOL AND 3-METHOXY-4-HYDROXYMANDELIC ACID

Fluorimetric method based on oxidation to vanillin with subsequent condensation with thiosemicarbazide.

The estimation of urinary 3-methoxy-4-hydroxymandelic acid (vanillin mandelic acid, VMA) involves its oxidation to 3-methoxy-4-hydroxybenzaldehyde (Vanillin) at an alkaline pH by sodium metaperiodate (Pisano, Crout and Abraham, 1962). After adjustment of the pH of the reaction mixture to 7.5 the vanillin is extracted by shaking with toluene and is returned to an aqueous medium by shaking the toluene extract with a solution of potassium carbonate. The absorption of the vanillin solution is then measured at 360 mμ. Ruthven and Sandler (1965) have used the same oxidation method for the estimation of 3-methoxy-4-hydroxyphenylethyleneglycol (MOPEG).

Goldenberg and White (1962) described the condensation of vanillin, at an alkaline pH, with thiosemicarbazide to give a compound with a high molar fluorescence. This method should enable the estimation of very small quantities of VMA or MOPEG, but previous work, in this laboratory (Guldberg, unpublished observations) has shown the method as described to be unreliable and so an attempt

was made to modify the procedure.

The coupling of vanillin with thiosemicarbazide Samples of vanillin in 1.0 ml 1M potassium carbonate (pH 11.5) in 15 ml glass stoppered test tubes were heated, in the dark, at 50°C for 30 mins with 0.5 ml of a 10 mg/ml solution of thiosemicarbazide. After cooling the samples to room temperature, the fluorescence developed from the vanillin was estimated on an Aminco-Bowman spectrophotofluorimeter at an activation wavelength of 375 mμ and a fluorescence wavelength of 460 mμ (uncorrected values). The tubes were returned to the waterbath at 50°C and their fluorescence was again measured after further 30 mins incubations, up to and including a total incubation time of 150 mins. The fluorescence produced from vanillin increased up to an incubation time of 60 mins after which it remained constant. Turbidity, giving rise to high reagent blanks, was observed in all tubes and was found to increase with time.

In an attempt to reduce this turbidity, the experiment was repeated using a 60 mins incubation with 0.5 ml of a 1 mg/ml solution of thiosemicarbazide. No fluorescence was observed even with concentrations of vanillin of 1 μg/ml. The turbidity formed in the tubes, however, was very much reduced which suggests that it was due to the thiosemicarbazide. Neither filtration through Whatman No 1 filter paper nor centrifugation for 5 mins at 2,200 g was effective in reducing the reagent blank. Centrifugation for 2 mins at 12,000 g however did reduce the turbidity, but it

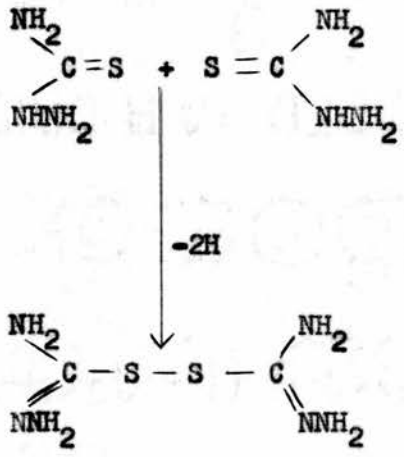


Fig. 2:2

Proposed oxidation product of thiosemicarbazide formed on heating it in alkaline solution.

reformed again on letting the solution stand. The formation of the turbidity could be prevented by the addition of either 0.1 ml of a 50 mg% w/v solution of ascorbic acid or cysteine to the incubation medium. This, however, also interfered with the coupling of vanillin with the thiosemicarbazide. The turbidity produced on heating thiosemicarbazide in alkaline solution may, therefore, be due to its oxidation to give an insoluble dimer as shown in Fig. 2:2.

The condensation of an aldehyde with hydrazine and hydrazine-like compounds is normally achieved in dilute acid solution (Schmidt, 1936; Georges, 1964). This may also reduce the possibility of oxidation of the thiosemicarbazide.

Samples containing vanillin in 1.0 ml 0.2M acetate buffer pH 4.0 in 15 ml glass stoppered test tubes were incubated at 50°C for 1 hour with 0.5 ml of a 10 mg/ml solution of thiosemicarbazide. The tubes were cooled to room temperature, adjusted to pH 10.0 with 0.4 ml 1M potassium carbonate and the fluorescence measured as previously described. The fluorescence produced from the vanillin was proportional to the concentration of vanillin in the original solution and the reagent blank was lower than before as no turbidity was produced in the tubes. The limit of sensitivity of the method was taken as the concentration of vanillin required to produce a fluorescence equal to the reagent blank and was found to be about 100 ng/ml. When the incubation was carried out at pH 4.0 the fluorescence produced from vanillin increased with time up to 1 hour, with a 25% reduction and an increase in the reagent blank after

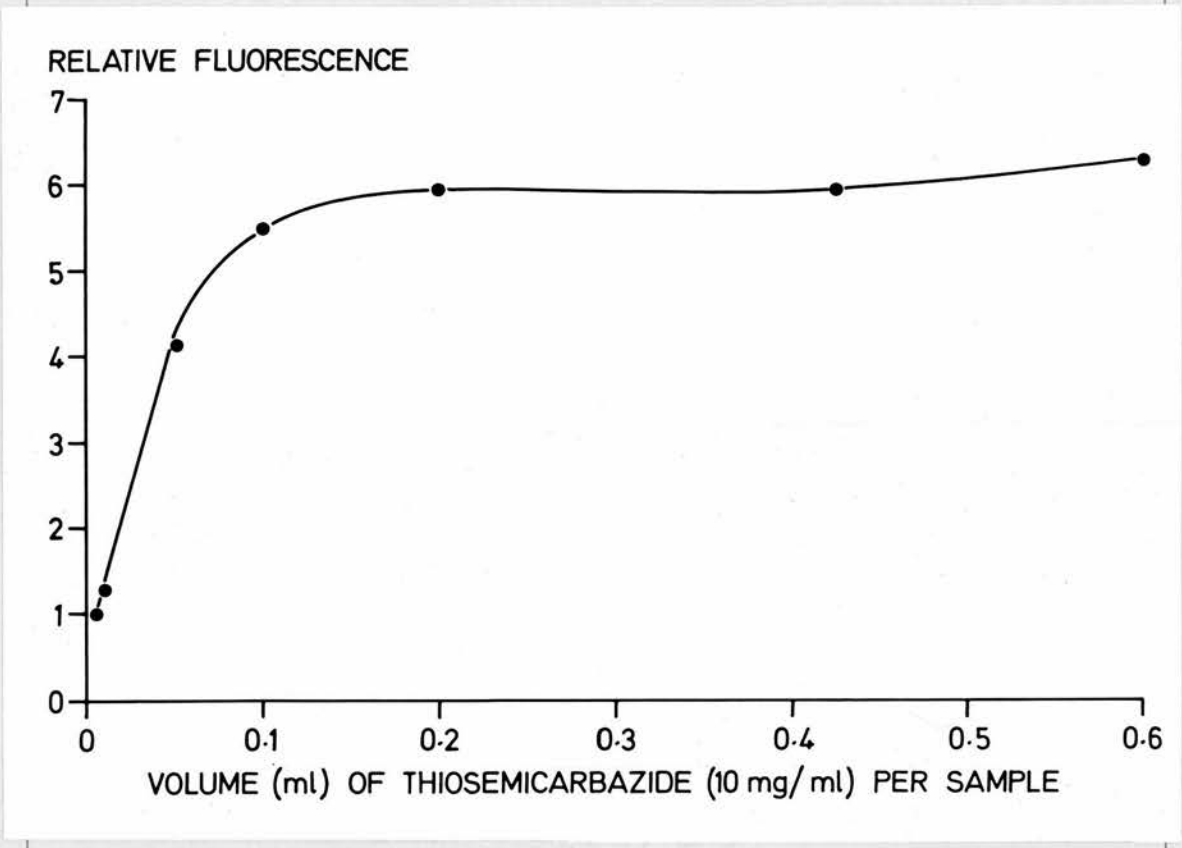


Fig. 2:3

Fluorescence from 200ng vanillin in 1 ml 0.2M acetate buffer pH 4.0 incubated at 50°C for 60 min with varying volumes of a 10 mg/ml solution of thiosemicarbazide. The fluorescence was estimated at an activation wavelength of 375 mμ and a fluorescence wavelength of 460 mμ. All readings are corrected for the fluorescence due to the reagents and also for differences in volume.

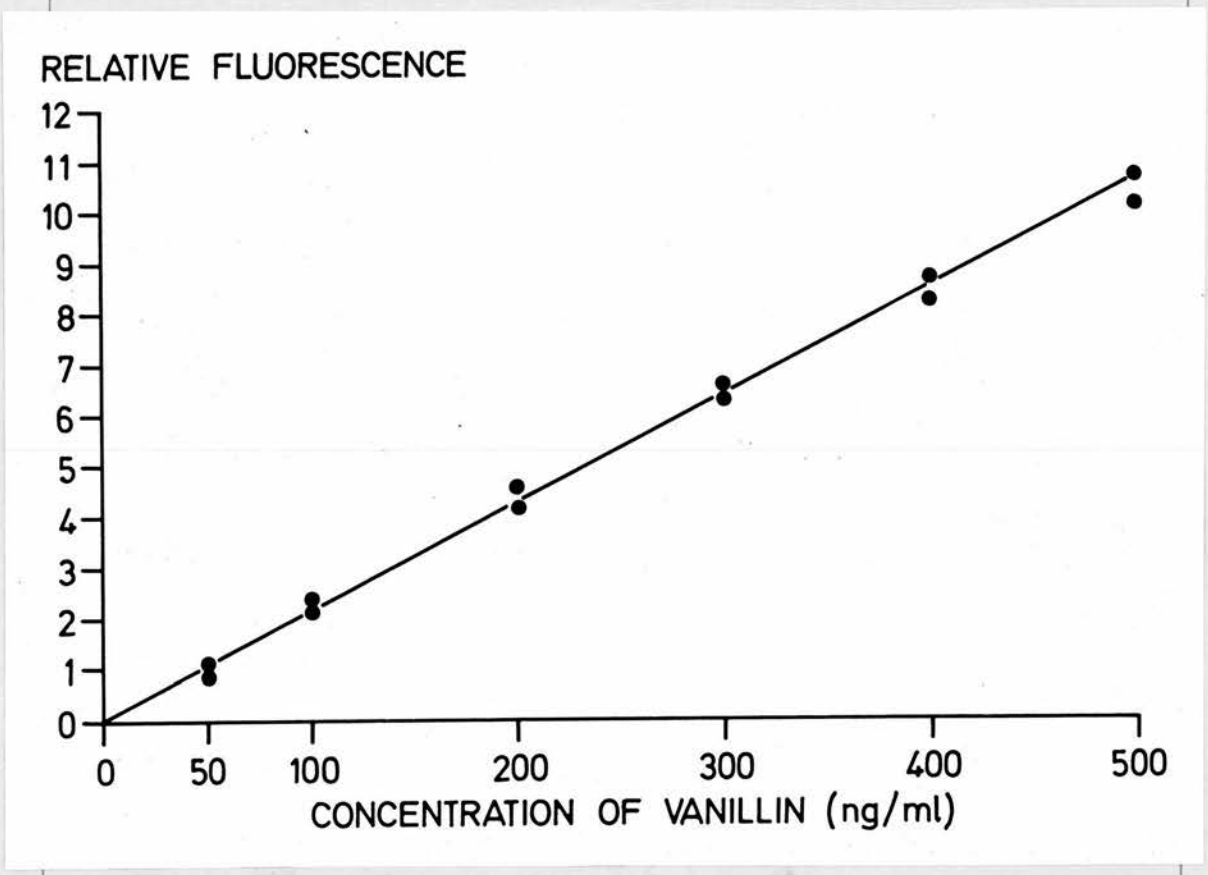


Fig. 2:4

Fluorescence obtained from 1 ml solutions of vanillin (ng/ml) when incubated at 50°C for 1 hr with 0.2 ml of a 10 mg/ml solution of thiosemicarbazide. The fluorescence was estimated at an activation wavelength of 375 mμ and a fluorescence wavelength of 460 mμ. The fluorescence of the reagent "blank" has been subtracted in each case.

3 hours. A 1 hour incubation was used in subsequent estimations.

The concentration of thiosemicarbazide in these determinations is in considerable excess of the stoichiometric amount required to react with the vanillin. It was found, however, that reduction of this to below 0.2 ml of a 10 mg/ml solution caused a decrease in the final fluorescence obtained from the vanillin (Fig. 2:3). The reagent blank increased with increased concentrations of thiosemicarbazide and so 0.2 ml of a 10 mg/ml solution of thiosemicarbazide was added to the 1 ml samples of vanillin in 0.2M acetate buffer in subsequent experiments.

Samples of vanillin in 1 ml acetate buffer at pH 4.0 were incubated in glass stoppered test tubes in a waterbath at 50°C for 1 hour in the dark with 0.2 ml of a 10 mg/ml solution of thiosemicarbazide. This gave a fluorophor with an activation wavelength of 375 mμ and a fluorescence wavelength of 460 mμ. The fluorescence was proportional to the concentration of vanillin and the limit of sensitivity of the method was 50 ng vanillin per ml of the original solution (Fig. 2:4).

Oxidation of VMA or MOPEG to vanillin The method described by Pisano et al., (1962) was used in an attempt to convert nanogram quantities of VMA to vanillin.

Samples containing between 100 ng and 1.0 μg of VMA in 1.0 ml 1M potassium carbonate in 15 ml glass stoppered test tubes were heated in a waterbath for 30 mins at 50°C with 0.05 ml 2% w/v sodium

metaperiodate. After cooling the samples to room temperature, excess metaperiodate was reduced to iodide by the addition of 0.05 ml 10% w/v sodium metabisulphite. The samples were adjusted to pH 7.5 by the addition of 0.3 ml 5M acetic acid and 0.6 ml 3M phosphate buffer pH 7.5 and any vanillin formed from the VMA extracted from this reaction mixture by shaking it for 5 mins with 10 ml toluene. After centrifugation at 2,000 g for 5 mins, 9.5 ml of the toluene extract was transferred to another 15 ml glass stoppered test tube and the vanillin returned to an aqueous phase by shaking the toluene extract with 1.2 ml 0.2M potassium carbonate. The mixture was then centrifuged at 2,000 g for 5 mins and 1.0 ml of the carbonate layer transferred to a clean 15 ml glass stoppered test tube and adjusted to pH 4.0 by the addition of 0.1 ml 5M acetic acid. This solution was incubated with thiosemicarbazide and the fluorescence estimated as previously described. The fluorescence produced was not proportional to the concentration of VMA in the original sample and the reagent blank was very high.

It was found that trace amounts of toluene added to samples containing vanillin interfered with the production of a fluorophor when incubated with thiosemicarbazide. The estimation was therefore modified in order to avoid the toluene extraction step.

Samples of between 100 ng and 1 µg of VMA in 1.0 ml 0.2M potassium carbonate were incubated with 0.05 ml 2% w/v sodium metaperiodate as previously described. After the addition of 0.05 ml 10% w/v sodium metabisulphite the samples were adjusted to pH 4.0 by

the addition of 0.1 ml 5M acetic acid and heated for 1 hour at 50°C with 0.2 ml of a 10 mg/ml solution of thiosemicarbazide. The fluorescence, estimated as before, was not proportional to the concentration of VMA in the original sample. This was thought to have been due to the interaction of the aldehyde group of the vanillin with the sodium metabisulphite to give a condensation compound which was dissociated to varying degrees by heating at pH 4.0. When 1.0 ml containing vanillin was condensed with 0.2 ml of 10 mg/ml thiosemicarbazide after the addition of 0.05 ml 2% w/v sodium metaperiodate and 0.05 ml 10% w/v sodium metabisulphite, the fluorescence produced was 40% lower than when the condensation was done in their absence. If, however, the sodium metabisulphite was not added a yellow coloration formed on incubating the sample with thiosemicarbazide. Adjustment of the sample to pH 4.0 before the addition of the metabisulphite decreased the reduction in fluorescence to 15% and no yellow coloration was observed. When a similar adjustment to pH 4.0 by the addition of the acetic acid was made before the metabisulphite addition in the estimation of VMA it resulted in an increase in the fluorescence produced from any one sample but did not improve the reproducibility of the method.

It thus seems that the oxidation of VMA, in the range 100 ng-1 µg to vanillin is not reproducible under the conditions used. A search for a more reliable method for the fluorimetric estimation of VMA and MOPEG gave rise to the one described below.

Ferric chloride method for the fluorimetric estimation of 3-methoxy-4-hydroxymandelic acid and 3-methoxy-4-hydroxyphenylethyleneglycol.

This method is a modification of that described by Sharman (1963) for the estimation of 3-methoxy-4-hydroxyphenylacetic acid (homovanillic acid, HVA).

To 0.5 ml of a sample containing VMA or MOPEG in a 1.5 ml conical polystyrene centrifuge tube (Eppendorff 'microlitre' reaction tube) was added 0.05 ml of a freshly prepared 1% w/v ferric chloride solution (by dilution of B.D.H. Analar 60% w/v ferric chloride solution) to every 10 ml of which had been added 0.2 ml of 2N NaOH. After exactly 2 mins in the dark, the ferric ions were precipitated from the reaction mixture by the addition of 0.05 ml 5N NaOH. The precipitate formed was separated by centrifugation at 12,000 g for 1 min. The supernatant fluid, 0.5 ml, was transferred to the microcuvette of a Zeiss spectrophotofluorimeter and the fluorescence of the fluorophor produced from the VMA or MOPEG was determined from the recorded activation spectrum at the wavelength of maximum activation of 325 mμ and at a wavelength of 430 mμ, the maximum for the fluorescence emission. These quoted wavelengths for the two maxima are uncorrected instrument values. A filter with a band pass of between 250 and 380 mμ was placed between the activation monochromator and the sample chamber. This reduced the Raman peak and the scatter peak when the spectrum was recorded from 250 mμ to

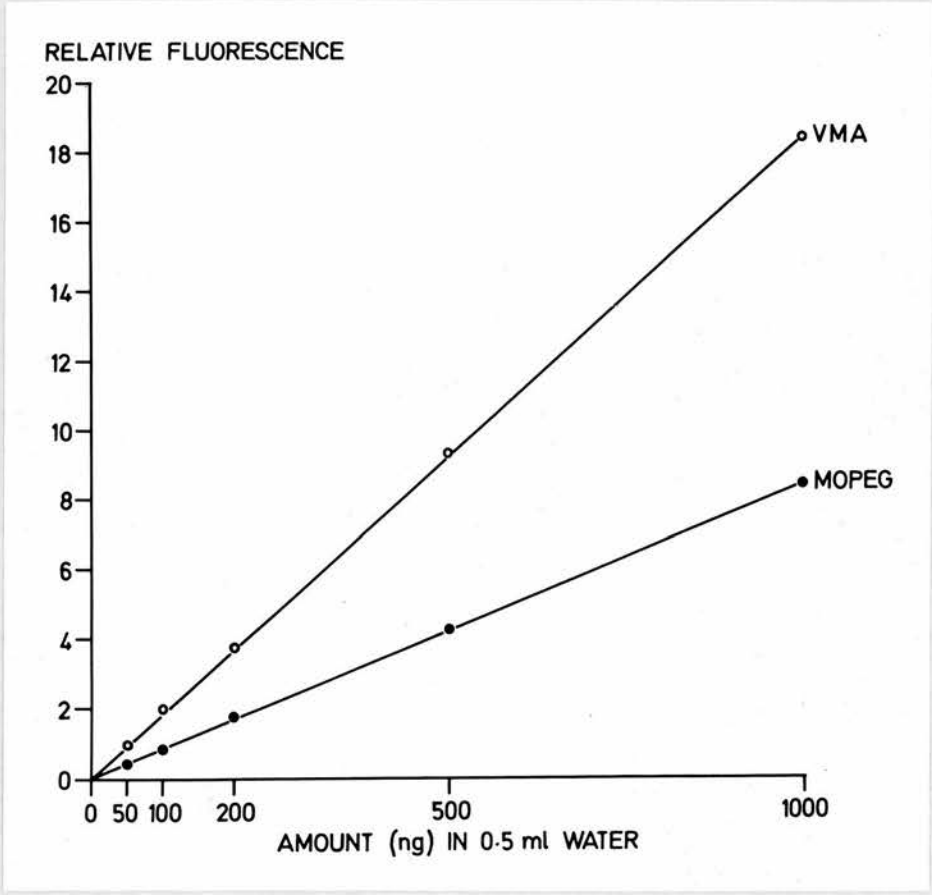


Fig. 2:5

Relative fluorescence of the fluorophor derived from various amounts of VMA and MOPEG by reaction with the ferric chloride reagent. The fluorescence of the reagent "blank" has been subtracted from each estimation.

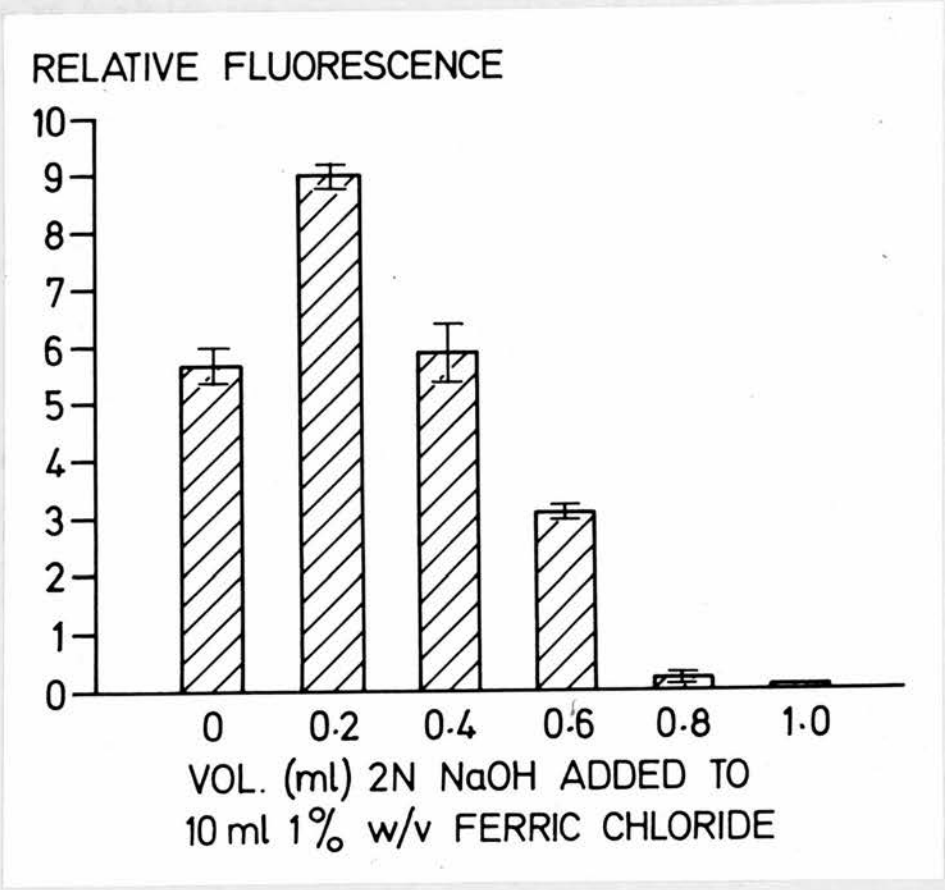


Fig. 2:6

The effect on the fluorescence of 500 ng MOPEG of adding varying volumes (ml) of 2N sodium hydroxide to 10 ml of 1% (w/v) ferric chloride in the formation of the oxidising reagent. Each column represents the mean $\pm$ the standard deviation of four estimations.

480 mμ (uncorrected values) and thus enabled the fluorescence peak to be seen more easily. The band pass of the activation monochromator when nominally set at a wavelength of 325 mμ was from 321 to 330 mμ at a 'slit width' setting of 1 mm. Because the dispersal of the light in this monochromator was obtained by a prism, the band pass increased continuously when the activation spectrum was recorded from the lower to the higher wavelength.

The slit width of the analysing monochromator was 2 mm giving a band pass of 10 mμ which remained constant at all wavelengths because light dispersal in this monochromator was produced by a diffraction grating.

The fluorescence produced was proportional to the concentration of MOPEG or VMA in the original sample; the fluorescence yield being greater for VMA than for MOPEG (Fig. 2:5).

Composition of the ferric chloride reagent. Fluorophor production from MOPEG or VMA was dependent on the amount of sodium hydroxide added to the 1% w/v ferric chloride solution (Fig. 2:6) and a reagent producing the maximum yield of fluorophor was obtained by adding 0.2 ml 2N sodium hydroxide to every 10 ml of 1% w/v ferric chloride. This produced a precipitate of ferric hydroxide which formed a dark reddish-brown colloidal solution on mixing and standing for about 10 mins. The reagent could be used either before or after the formation of the colloidal solution with no detectable difference in the fluorescence intensity.

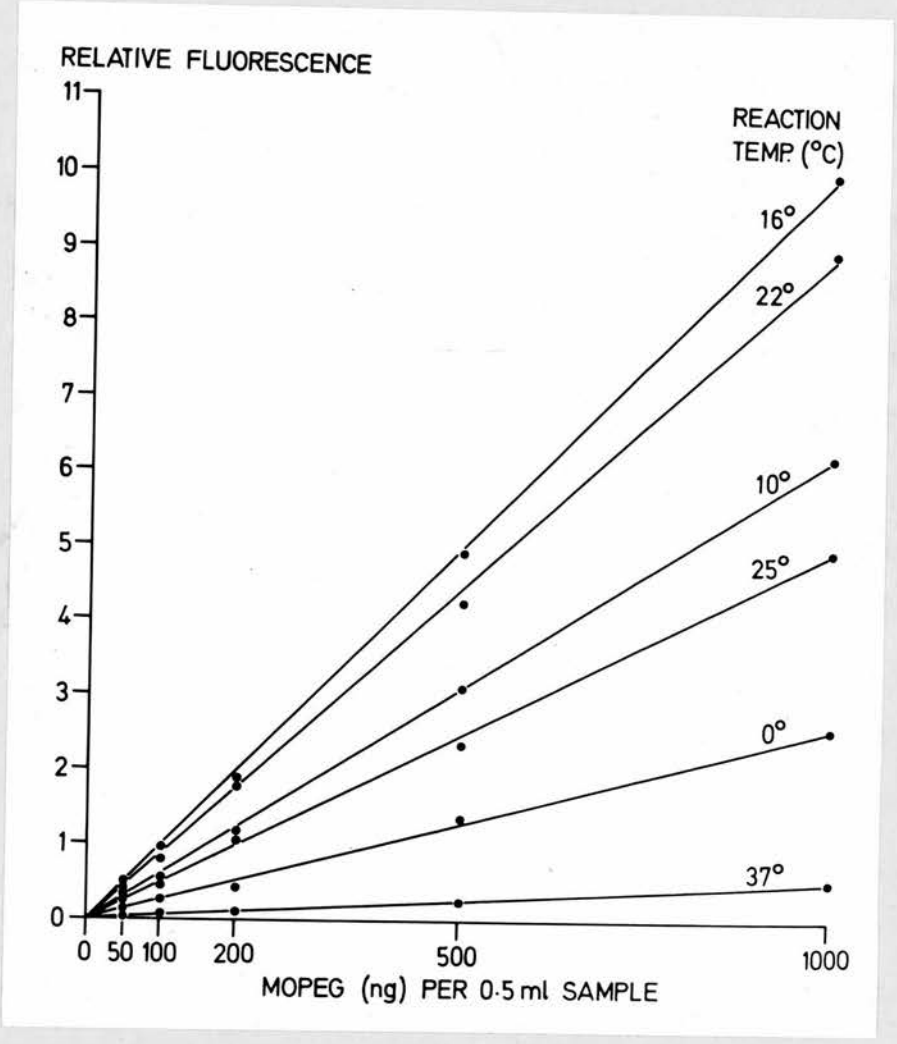


Fig. 2:7

The effect on the fluorescence intensity of MOPEG of carrying out the fluorophor-producing reaction at different temperatures.

Temperature of the reaction with the ferric chloride reagent. Alteration of the temperature at which the reaction of VMA or MOPEG with the ferric chloride reagent was carried out markedly changed the fluorescence yield from both of the compounds (Fig. 2:7). The reaction was normally done at room temperature (approx. 20°C) except on hot summer days when the ambient temperature could rise as high as 27°C. On such occasions all reagents were cooled in running tap water (approx. 14°C) and the fluorophor from VMA or MOPEG was produced in the 1.5 ml polystyrene reaction tubes cooled in a metal block through which tap wator was circulating .

Centrifugation. The reaction of the VMA or MOPEG with the ferric chloride reagent was terminated by precipitating the ferric ions with 0.05 ml 5N NaOH. The ferric hydroxide thus formed was removed by centrifugation. The fluorescence intensity, as measured in the fluorimeter, was dependent on the efficiency of this centrifugation step.

Standard solutions of 200, 500 and 1,000 ng of MOPEG in 0.5 ml deionized distilled water were reacted with the ferric chloride reagent as described above. After the addition of 0.05 ml of 5N NaOH the solution was centrifuged at 2,000 g for 1 min and the fluorescence of the supernatant estimated at an activation wavelength of 325 mμ and a fluorescence wavelength of 430 mμ (uncorrected values). The solution was then placed in a 1.5 ml polystyrene reaction tube, centrifuged at 12,000 g and the

fluorescence of the supernatant estimated as before. A reagent blank, consisting of 0.5 ml deionized distilled water, was treated in the same way as the standards.

The fluorescence was markedly increased by recentrifugation of the reaction mixture at the higher acceleration (Table 2:1). This increase was thought to be due to a reduction in the particulate matter in the supernatant from the first centrifugation, causing a reduction in the internal reflection of the fluorescence light.

Stability of the fluorophor. The fluorophor produced from VMA or MOPEG was stable, after the removal of the ferric ions from the reaction mixture, for up to 30 mins when kept in the dark. If exposed to light producing maximal activation of the fluorophor (325 mμ) the intensity of the emitted fluorescence diminished very rapidly.

Because of the findings regarding the stability of the fluorophor the following routine was imposed. The number of samples treated concurrently was restricted to six. The reaction with the ferric chloride reagent was carried out under subdued lighting conditions i.e. in the absence of sunlight or direct illumination from artificial light. After precipitation of the ferric ions and centrifugation, the supernatant fluid was kept in the dark until fluorimetry could be performed not more than 20 mins later. For each sample, the activation spectrum was recorded twice; the relative fluorescence as measured from the second

Table 2:1

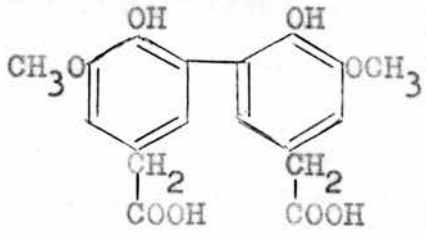
The effect on the fluorescence of MOPEG by centrifuging the reaction tube at 2,000 or 12,000 g to remove the precipitate of ferric hydroxide.

Concentration of MOPEG (ng/0.5 ml)	Relative fluorescence Centrifuged at 2,000 g	$R_e$centrifuged at 12,000 g	% increase
200	96	176	83
500	232	422	82
1000	465	850	83

Fig 2:8.

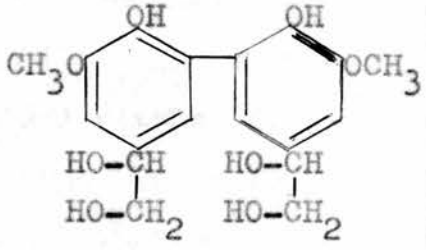
Proposed fluorophor from:-

HVA



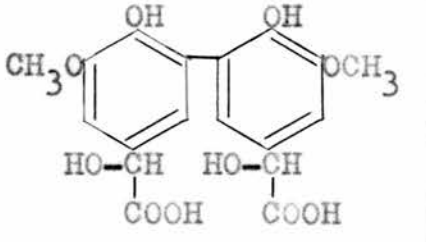
2-2'-dihydroxy-3-3'-dimethoxy-biphenyl-5-5'-diacetic acid

MOPEG



2-2'-dihydroxy-3-3'-dimethoxy-biphenyl-5-5'-diethyleneglycol

VMA



2-2'-dihydroxy-3-3'-dimethoxy-biphenyl-5-5'-diglycollic acid

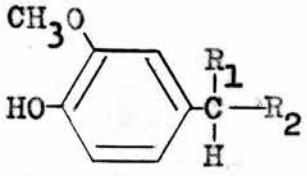
spectrum recorded was always slightly less than that from the first.

Specificity of method. The method for the estimation of VMA and MOPEG will produce a fluorophor, with the same activation and fluorescence characteristics, from other O-methylated catechol derivatives such as metanephrine, normetanephrine, 3-methoxytyramine, 3-methoxy-4-hydroxyphenylacetic acid (homovanillic acid, HVA) and 3-methoxy-4-hydroxyphenylethanol (MOPET). The relative fluorescence of 500 ng of these compounds taken through the method is shown in Table 2:2.

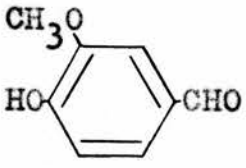
Nature of reaction. The nature of the reaction for the production of a fluorophor from VMA and MOPEG is not altogether understood. It is very probably similar to the reaction for the production of a fluorophor by the oxidation of HVA with ferricyanide (Andén, Roos and Werdinius, 1963a) or with ferric chloride (Sharman, 1963). Corrodi and Werdinius (1965) have isolated a fluorophor formed by the oxidation of HVA and have established its structure as 2,2'-dihydroxy-3,3'-dimethoxybiphenyl-5,5'-diacetic acid (Fig. 2:8). A similar oxidation reaction would produce 2,2'-dihydroxy-3,3'-dimethoxybiphenyl-5,5'-diethyleneglycol from MOPEG, and 2,2'-dihydroxy-3,3'-dimethoxybiphenyl-5,5'-diglycollic acid from VMA (Fig. 2:8).

TABLE 2:2

The fluorescence developed at an activation wavelength of 325mμ and an emission wavelength of 430mμ by taking 500ng of some O-methylated catechol derivatives through the ferric chloride method for the development of a fluorophor from 3-methoxy-4-hydroxyphenylethyleneglycol.



R_1	R_2	3-methoxy-4-hydroxy-	Relative Fluorescence
-OH	$-CH_2OH$	-phenylethyleneglycol (MOPEG)	100
-H	$-CH_2OH$	-phenylethanol (MOPET)	14
-OH	-COOH	-mandelic acid (vanillin mandelic acid, VMA)	172
-H	-COOH	-phenylacetic acid (homovanillic acid, HVA)	228
-OH	$-CH_2NHCH_3$	-phenyl-N-methylethanolamine (metanephrine)	58
-OH	$-CH_2NH_2$	-phenylethanolamine (normetanephrine)	52
-H	$-CH_2NH_2$	-phenylethylamine (3-methoxytyramine)	277



-benzaldehyde (vanillin) 0

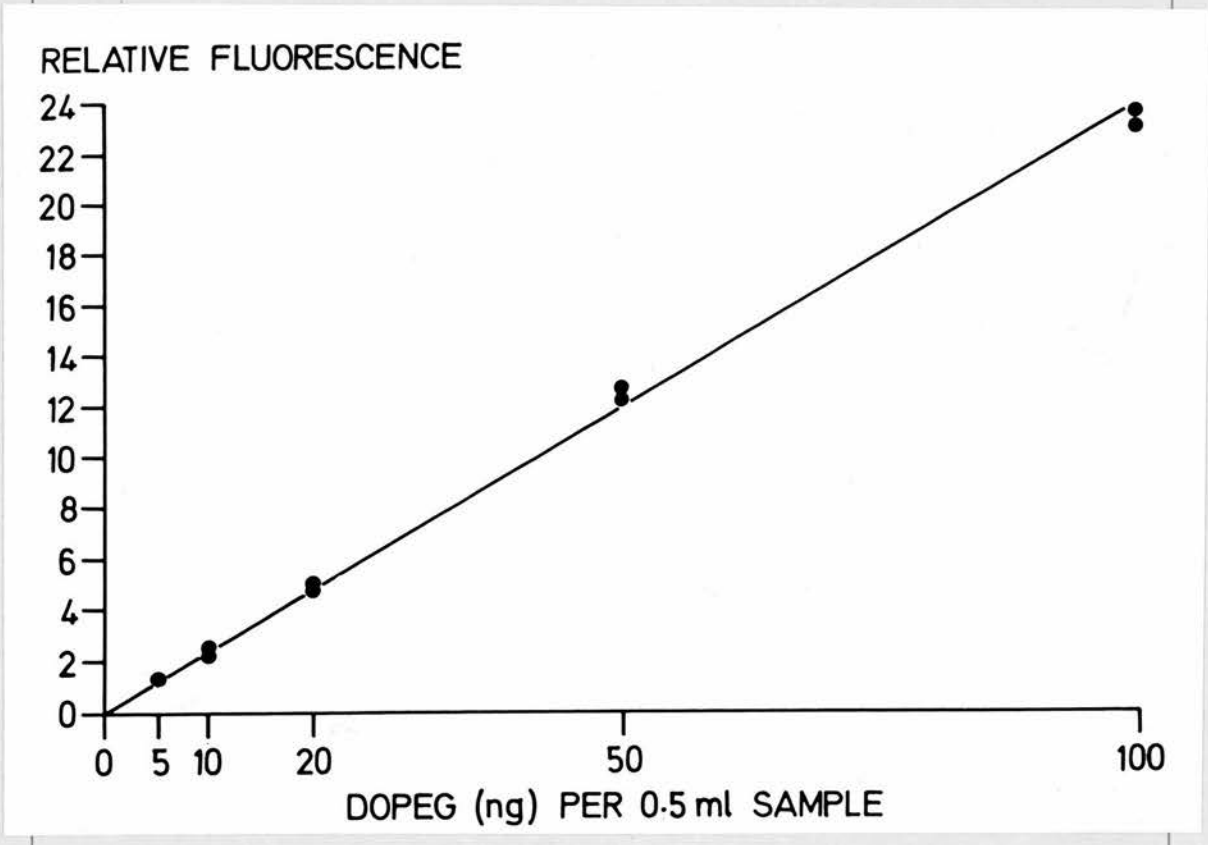


Fig. 2:9

Relation between relative fluorescence intensity and the quantity of DOPEG (ng/sample) after reaction with ethylenediamine. The fluorescence of the reagent blank has been subtracted in each case.

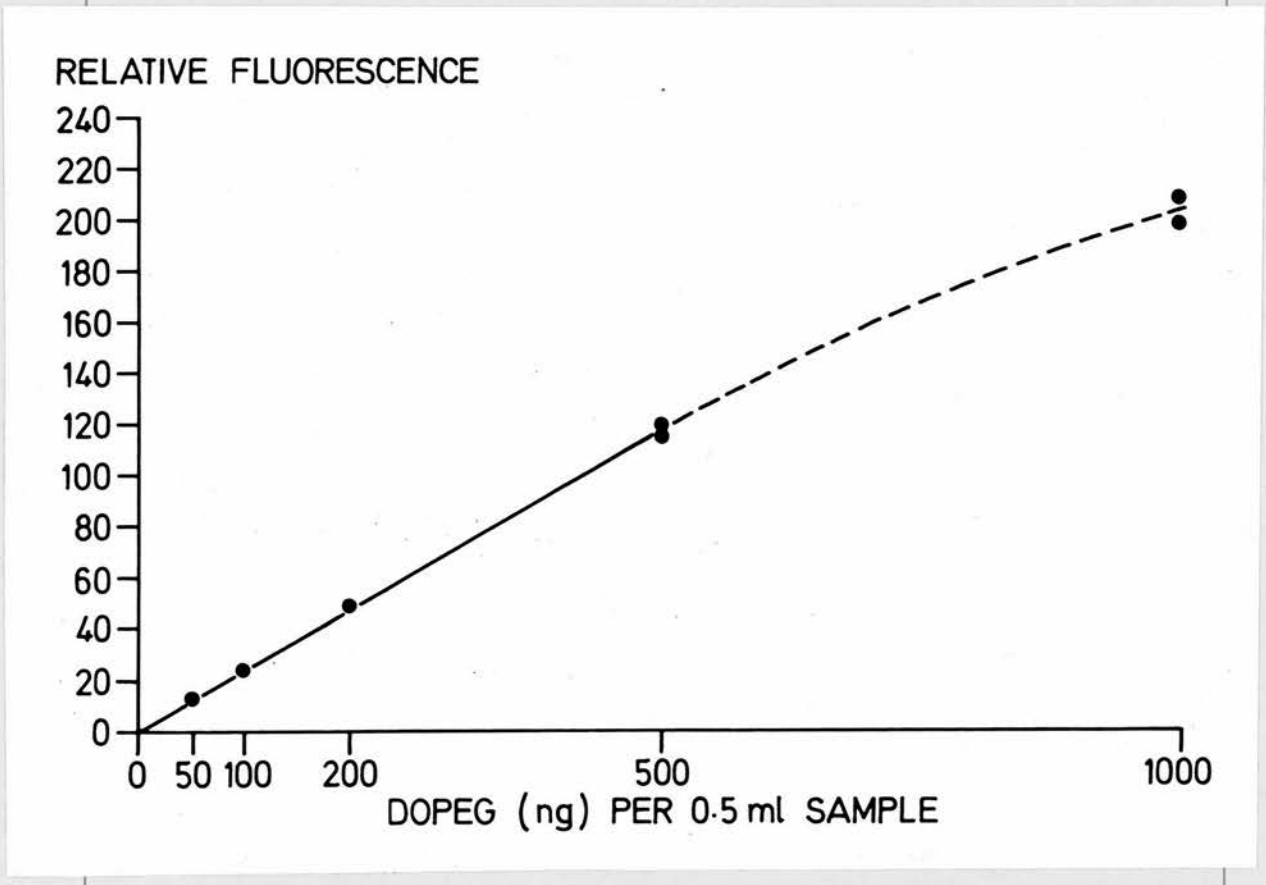


Fig. 2:10

Relation between relative fluorescence intensity and the quantity of DOPEG (ng/sample) after reaction with ethylenediamine. The fluorescence of the reagent blank has been subtracted in each case.

THE FLUORIMETRIC ESTIMATION OF 3,4-DIHYDROXYPHENYLETHYLENEGLYCOL

The method used for the estimation of 3,4-dihydroxyphenylethyleneglycol (DOPEG) was a modification of that described by Rosengren (1960) for the determination of 3,4-dihydroxyphenylacetic acid (DOPAC).

To a 0.5 ml sample containing DOPEG in deionized distilled water in a 1.5 ml polystyrene reaction tube with a snap closure was added 0.2 ml of a freshly prepared ethylene diamine: ammonium chloride mixture (twice distilled ethylene diamine: 4M ammonium chloride, 1:13 v/v) and the resultant solution heated in a heating block (Eppendorff 'microlitre' apparatus) at 56°C for 30 mins. After rapid cooling to room temperature, 0.5 ml of the solution was transferred to the microcuvette of a Zeiss spectrophotofluorimeter and the fluorescence of the fluorophor produced from the DOPEG was determined from the recorded activation spectrum at the wavelength of maximum activation of 410 mμ and at a wavelength of 510 mμ, the maximum for the fluorescence emission. These quoted wavelengths for the two maxima are uncorrected instrument values. The band pass of the prism activation monochromator when nominally set at 410 mμ was from 405.4 mμ to 415 mμ (0.5 mm slit width) and that of the grating fluorescence monochromator was 10 mμ for all wavelengths (2 mm slit width). The fluorescence developed was proportional to the concentration of DOPEG in the original sample over the range 5 to 500 ng in the 0.5 ml of deionized distilled water (Figs. 2:9 and 2:10). Above 500 ng there was slight self quenching of the

fluorescence of the fluorophor from DOPEG. The sensitivity of the method, taken as the amount of DOPEG required to produce a fluorescence equal to the 'reagent blank' was, in 'pure' solution in deionized distilled water, 2 ng/0.5 ml sample.

Stability of the fluorophor. The fluorescence of the fluorophor produced from DOPEG was stable for at least 1 hour if kept in the dark. When, however, it was exposed to its activating light at 410 mμ the fluorescence at 510 mμ decreased rapidly.

Specificity of reaction. This reaction is not specific for DOPEG as other compounds with a catechol grouping will react with ethylene diamine to produce a fluorophor. These include adrenaline and noradrenaline (Weil-Malherbe and Bone, 1952), dopamine (Weil-Malherbe and Bone, 1957), DOPAC (Rosengren, 1960), 3,4-dihydroxymandelic acid and 3,4-dihydroxyphenylethanol (Ashcroft, Crawford, Dow and Guldberg, 1968).

APPLICATION OF FLUORIMETRIC METHODS TO THE ESTIMATION OF 3,4-DIHYDROXYPHENYLETHYLENEGLYCOL AND 3-METHOXY-4-HYDROXYPHENYL-ETHYLENEGLYCOL IN BODY FLUIDS AND TISSUES.

As the fluorimetric methods for the estimation of DOPEG and MOPEG are not specific for these compounds and will form fluorophors from other substances present in biological extracts, it was necessary to effect a partial purification of the two glycols prior to their estimation.

Extraction of MOPEG and DOPEG from aqueous solution. As MOPEG and DOPEG are polar compounds they are unlikely to be easily extracted from aqueous solution by organic solvents. They also contain no easily ionizable groups and their extraction might therefore be expected to be unaffected by the pH of the aqueous phase, provided it is below that required to ionize the phenolic groups.

In order to reduce the polarity of MOPEG and DOPEG, Sharman (1969) formed their acetyl derivatives which he extracted into methylene dichloride. The same method was used in the present study.

Samples containing MOPEG and DOPEG in 5.0 ml deionized distilled water in 30 ml glass stoppered test tubes were acetylated by the addition of 0.35 ml acetic anhydride and 0.7 g sodium bicarbonate to each sample. The reaction was allowed to proceed, with frequent shaking of the reaction mixture, for 10 mins when a further 0.35 ml of acetic anhydride and 0.7 g sodium bicarbonate was added. After a further 20 mins, during which time the reaction mixture was

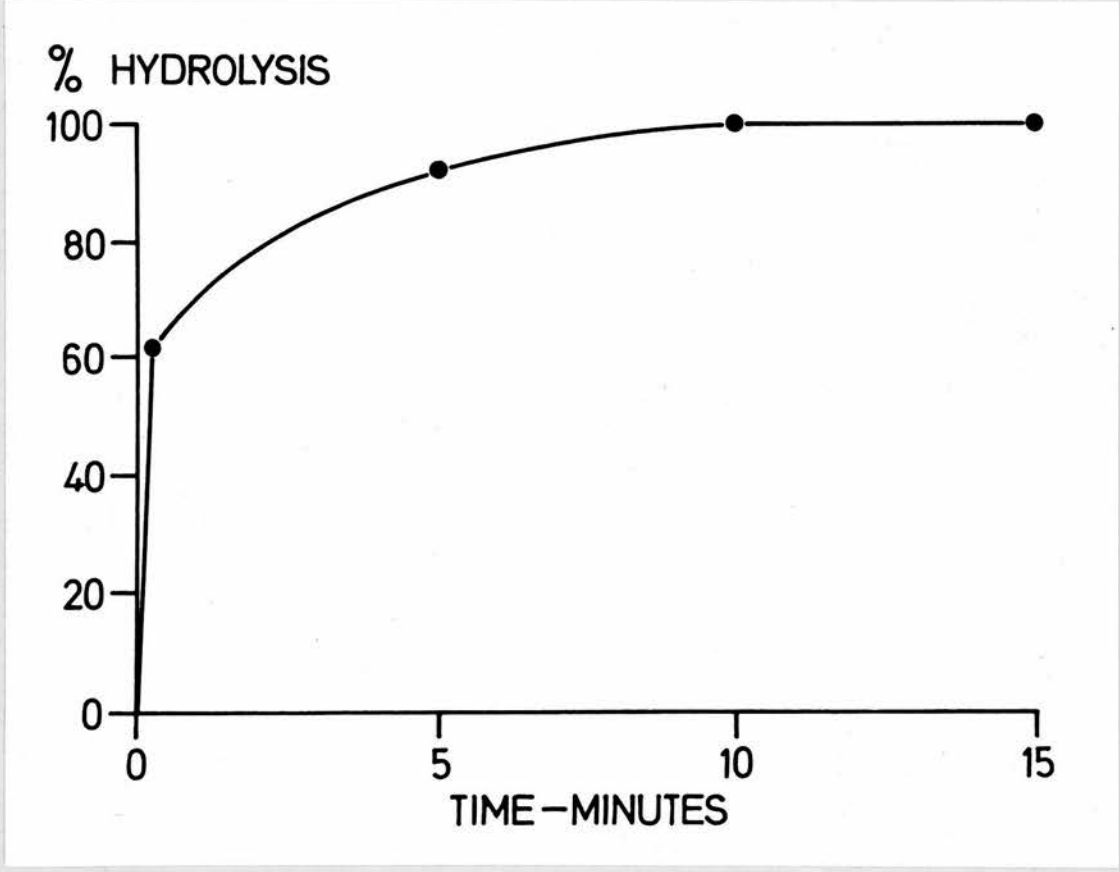


Fig. 2:11

Relation between the time of incubation (min) of acetyl MOPEG at room temperature in 0.1N NaOH and the percentage hydrolysis.

again frequently agitated, the acetylated glycols were extracted from aqueous solution by shaking for 5 mins with 15 ml of methylene dichloride (B.D.H. laboratory reagent; distilled twice). The phases were separated by centrifugation at 2,000 g for 5 mins and as much as possible of the bottom organic layer was passed through a siliconed filter paper (Whatman IPS) into a 100 ml round-bottomed flask and the filter paper was washed with approximately 2 ml methylene dichloride. The extraction was repeated with a further 15 ml of the organic solvent and the methylene dichloride from the second extraction, after filtration, pooled with that from the first. The combined extracts were blown to dryness at room temperature under a stream of nitrogen. The residue was dissolved in 1.0 ml deionized distilled water and transferred to a 1.5 ml polystyrene reaction tube for the estimation of MOPEG and DOPEG.

As the method used for the estimation of MOPEG does not produce a fluorophor from acetyl MOPEG, the latter substance had to be deacetylated before oxidation with the ferric chloride. A 0.5 ml portion of the extract was made alkaline by the addition of 0.05 ml 1N sodium hydroxide. Hydrolysis occurred at room temperature and reached a maximum after 10 mins (Fig. 2:11). After the addition of 0.05 ml 1N HCl the sample was adjusted to between pH 6.5 and 7.5 (glass electrode) with 0.1N HCl or 0.1N NaOH. If the pH of the sample was outside this range the fluorescence yield was very much reduced. After the deacetylation and pH adjustment, the fluorophor

was developed in the usual way.

There was no necessity to deacetylate the acetyl DOPEG as it was estimated, with the same molar fluorescence yield, by the method used for DOPEG. Deacetylation of the acetyl DOPEG probably occurs on heating it at 56°C for 30 mins with the ethylene diamine: ammonium chloride mixture.

Of the 500 ng of MOPEG in the original sample, 80 ± 2% (mean ± standard deviation: 4 estimations) was recovered in the final aqueous extract, and of 500 ng of DOPEG, 72 ± 3% (mean ± standard deviation: 4 estimations) was extracted by the methylene dichloride. The reagent blank, obtained by taking 5.0 ml of deionized distilled water through the method, was very large in both cases, very severely limiting the sensitivity of the method.

Chromatographic separation of acetyl MOPEG and acetyl DOPEG. The method used for the estimation of acetyl MOPEG or of acetyl DOPEG is specific to that acetylated glycol and will not produce a fluorophor from the other acetylated glycol. It should thus be possible to estimate either of the glycols when they are both present in the same sample. This, however, would reduce the sensitivity of the method of estimation as the final extract containing the two glycols would have to be divided into two portions; one for the estimation of acetyl MOPEG and one for the estimation of acetyl DOPEG. For this reason and also in an attempt to reduce the high reagent

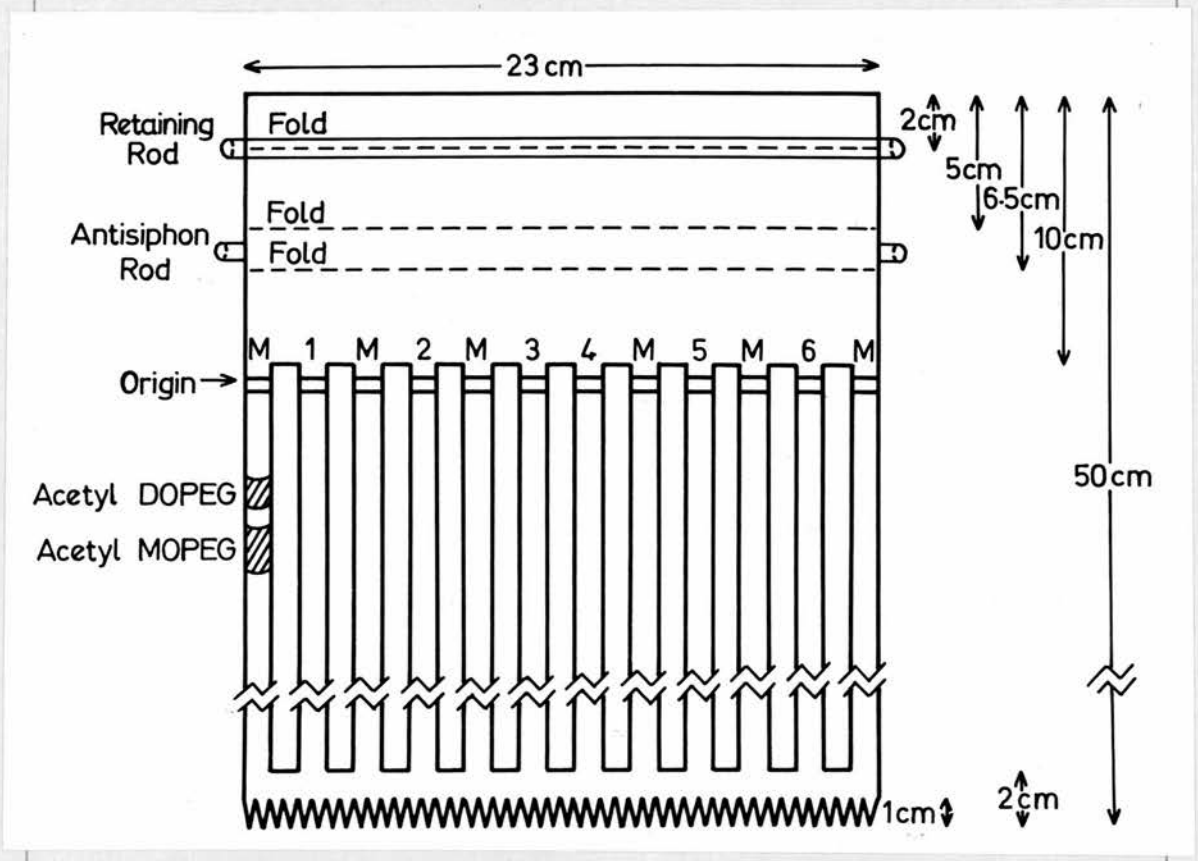


Fig. 2:12

Dimensions of the chromatography paper used for the descending chromatography of acetyl DOPEG and acetyl MOPEG.

blank obtained from the acetylation procedure, it was decided to examine the practicability of the chromatographic system described by Sharman (1969) for the quantitative separation of these two substances, as a desirable preliminary to their estimation.

Sharman (1969) separated acetyl MOPEG and acetyl DOPEG on Whatman No 50 paper which had previously been washed with sodium hydroxide and distilled water. Shortly after starting this work, W & R Balston Ltd, ceased production of Whatman No 50 chromatography paper and so another similar acid hardened paper, Whatman No 542, was used in its place.

Pieces of Whatman No 542 chromatographic paper, 23 cm wide and 54cm long, were rolled up and placed in approximately 1 litre of 2N sodium hydroxide in a 1 litre measuring cylinder, which was covered with paraffin film (Parafilm) to prevent the entry of dust. After 3 days, during which time the contents of the cylinder were occasionally mixed, the sodium hydroxide was decanted off and the paper rinsed in the cylinder with six changes of deionized distilled water. The cylinder was left full of deionized distilled water which was changed every working day for the next two weeks, after which time the paper was transferred to a chromatography tank where it was subjected to descending chromatography in deionized distilled water for at least 7 days. On the day before it was to be used, the paper was removed from the chromatography tank, dried for 1 hour at 40°C in an oven and cut in 1.0 cm strips as shown in Fig. 2:12.

Samples containing MOPEG or DOPEG in 5 ml deionized distilled water were acetylated and extracted with methylene dichloride as previously described. The residue in the 100 ml round bottomed flask, obtained after evaporating the methylene dichloride extract to dryness, was dissolved in 0.3 ml methanol and transferred by replicate applications of the solution to the centre of the origin line of the chromatographic strips (Fig. 2:12). The application was made, under a stream of nitrogen, to an area less than 1 cm in diameter. Care was taken to avoid extension of the spot area to the margins of the paper strip, as the substance ran unevenly if in contact with the edges of the strip. The round bottomed flask was washed out with a further 0.3 ml of methanol which was applied to the same spot as the first methanol extract. Samples or 'extraction blanks', which were obtained by taking 5 ml of deionized distilled water through the acetylation and extraction process, were applied to strips 1-6 (Fig. 2:12) and markers of authentic acetyl MOPEG and acetyl DOPEG were applied to the marker strips (M in Fig. 2:12). The markers were prepared by acetylating a solution of 10 mg of MOPEG or DOPEG in 5 ml deionized distilled water and extracting the acetylated glycols with methylene dichloride as previously described. The residue left after evaporation of the methylene dichloride extract to dryness in the round bottomed flask was dissolved in 1.0 ml 50% v/v methanol. Approximately 1 µl of this solution (about 10 µg of glycol) was applied to each marker strip.

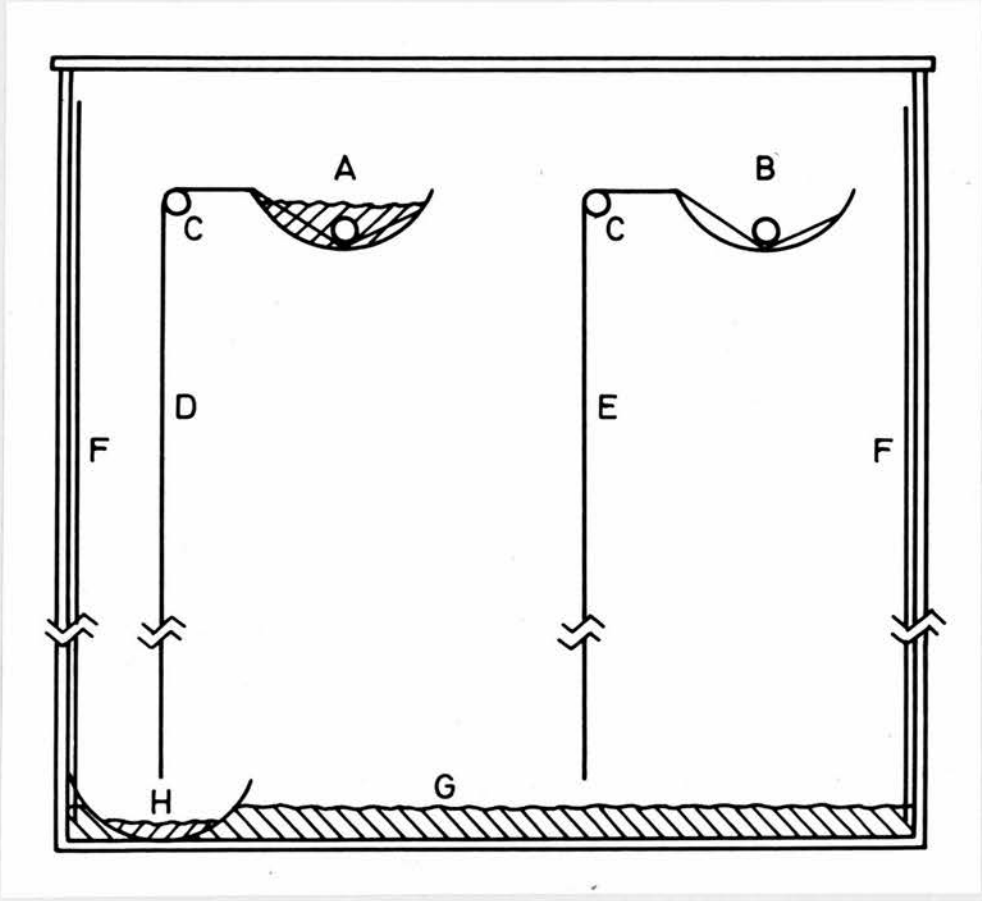


Fig. 2:13

Arrangement of the contents of the chromatography tank used for the chromatography of the acetylated glycols.

A - Trough containing aqueous phase of solvent system
B - Trough for organic phase of solvent system.
C - Anti-siphon rods.
D - Sheet of Whatman No 1 filter paper soaked with aqueous phase of solvent system.
E - Sample chromatogram.
F - Lining paper (Whatman No 1 filter sheet) soaked with the organic phase of the solvent system.
G - Organic phase of the solvent system.
H - Trough to catch aqueous phase dripping off the bottom of D.

Marker solutions were stable for up to 30 weeks if stored at -20°C.

The chromatogram was equilibrated overnight in an all-glass chromatography tank with both the organic and aqueous phases of a modified (Sharman; 1969) Bush "C" solvent system (Bush; 1952). The solvent system contained toluene: ethyl acetate: methanol: water, in the proportions 10:2:5:5 by volume which were shaken together in a 1 litre separating funnel and the two phases allowed to separate. The upper organic phase was run down the sheets of filter paper (Whatman No 1) lining the tank (F in Fig. 2:13) and formed a layer of solvent at the foot of the tank (G in Fig. 2:13). The lower, aqueous, phase was placed in a trough at the top of the tank (A in Fig. 2:13) from which was suspended a sheet of Whatman No 1 chromatography paper 50 cm long and 23 cm wide (D in Fig. 2:13). The sample chromatogram (E in Fig. 2:13) was suspended from another trough at the top of the tank (B in Fig. 2:13). The glass cover of the tank was sealed on with a paste made by heating 20 g starch in 70 g glycerol, and was held tight with screw clamps.

After equilibration overnight the chromatogram was developed for 5 hours in the descending direction in the organic phase of the modified Bush "C" solvent. The door of the room in which the chromatogram was being developed was kept closed in order to prevent draughts producing an uneven temperature across the tank, as this caused the substance on the strips near the edge of the tank to flow more slowly than those in the middle. When the chromatogram had been

developed it was removed from the tank, dried in a stream of air in a fume cupboard for 30 mins and the strips containing the samples cut from the rest of the chromatogram. The acetylated glycols on the marker strips were visualised by spraying with 5% w/v sodium hydroxide in 50% methanol, and, after the strips had dried, diazotised p-nitroaniline. The alkali deacetylated the compounds and allowed the free phenolic glycols to react with the diazotised p-nitroaniline. The diazotised p-nitroaniline was prepared by adding 5% w/v sodium nitrite to a saturated solution of p-nitroaniline in 0.1N HCl, until a colourless solution was obtained. Acetyl DOPEG gave a blue-green spot the centre of which was approximately 4.5 cm from the origin while acetyl MOPEG produced an intense purple colour approximately 6.5 cm from the origin (Fig. 2:12).

The parts of the sample chromatograms corresponding to acetyl DOPEG and acetyl MOPEG in the marker strips were cut out, rolled into a cylindrical spiral and eluted by shaking them for 30 mins with 0.7 ml deionized distilled water in a 5 ml glass stoppered test tube. The acetyl DOPEG or acetyl MOPEG in a 0.5 ml portion of the appropriate eluate was estimated as previously described.

Known amounts of acetyl MOPEG or acetyl DOPEG applied to the origin of a chromatogram on alkali washed Whatman No 542 paper and developed in the organic phase of the modified Bush "C" solvent gave a greater than 95% recovery for acetyl MOPEG and a greater than 92% recovery for acetyl DOPEG when they were eluted and estimated

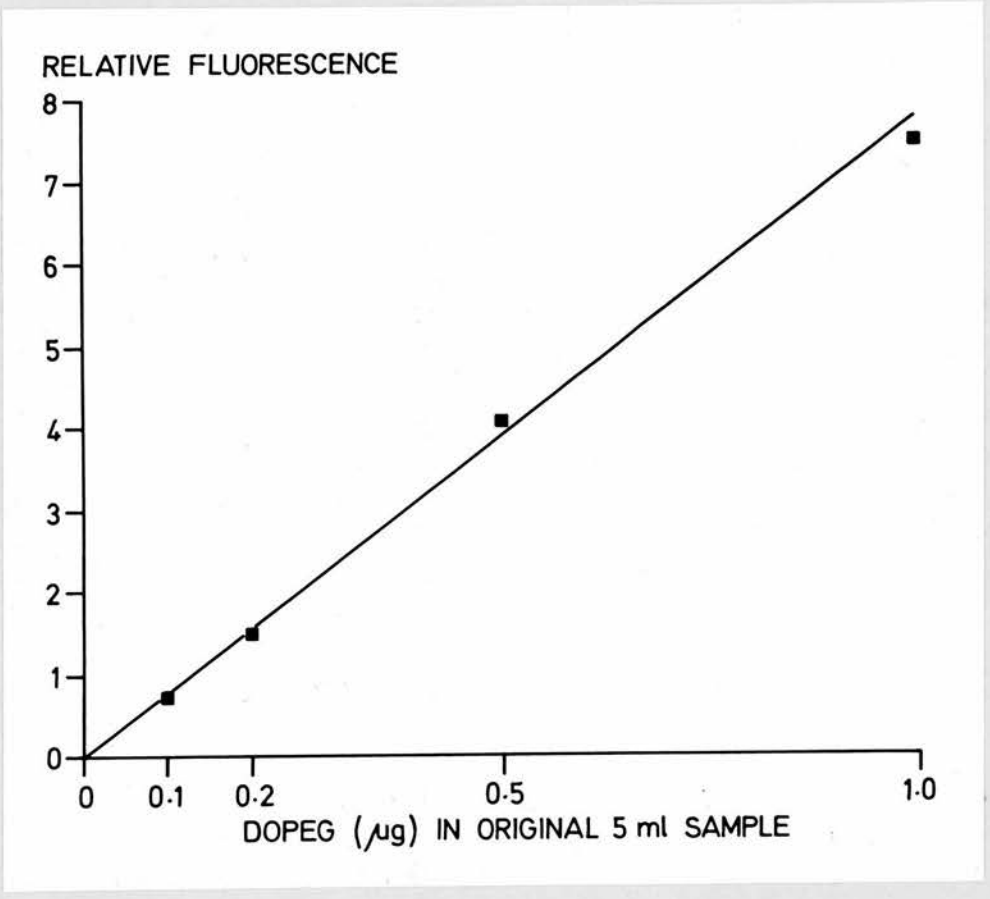


Fig. 2:14

Relation between the relative fluorescence intensity and the amount (µg) of 3,4-dihydroxyphenylethyleneglycol (DOPEG) in 5 ml of water when taken through the acetylation procedure, chromatography and fluorophor-producing reaction. The fluorescence of the reagent blank has been subtracted in each case.

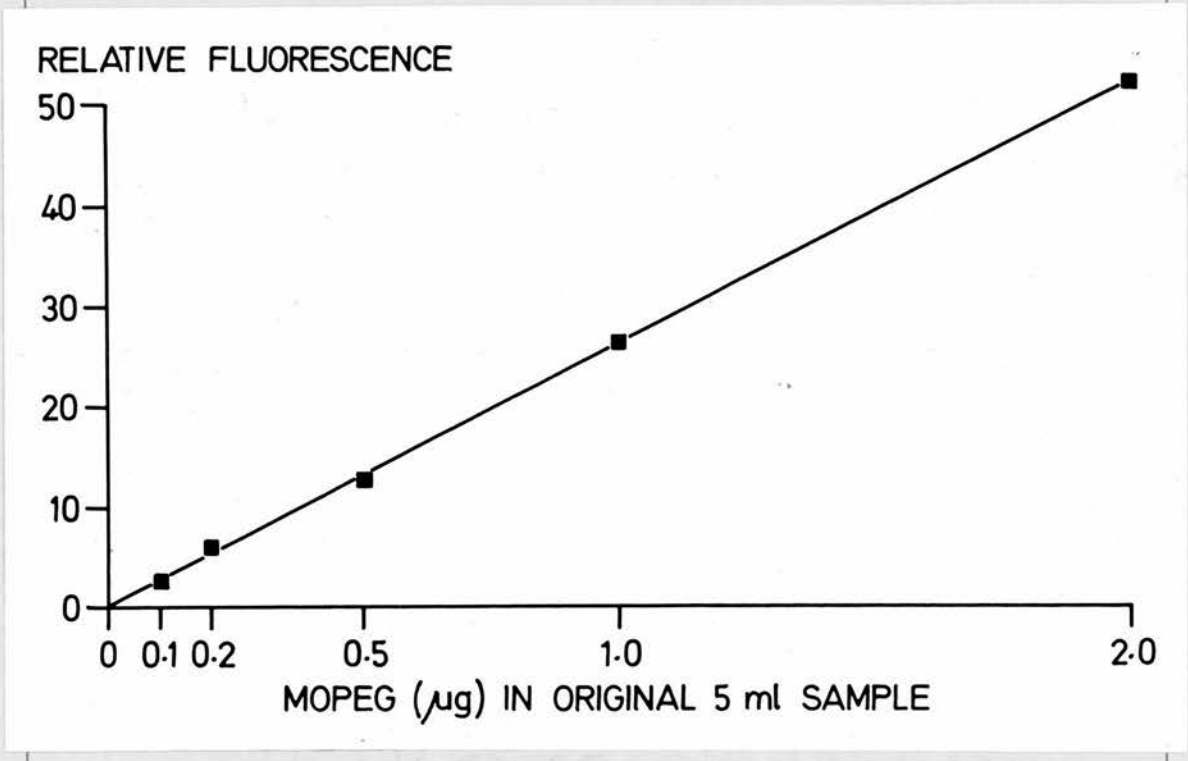


Fig. 2:15

Relation between the relative fluorescence intensity and the amount (µg) of 3-methoxy-4-hydroxyphenylethyleneglycol (MOPEG) in 5 ml of water when taken through the acetylation procedure, chromatography and fluorophor-producing reaction. The fluorescence of the reagent blank has been subtracted in each case.

as described above (Table 2:3).

The fluorescence produced in the final eluate from the chromatogram was proportional to the amount of DOPEG (Fig. 2:14) or MOPEG (Fig. 2:15) in the original 5 ml deionized distilled water. The recovery of DOPEG through the method was 61 $\pm$ 3 (mean percentage recovery $\pm$ standard deviation: 4 estimations) and for MOPEG was 63 $\pm$ 4 (mean percentage recovery $\pm$ standard deviation: 16 estimations). The recoveries for both alcohols were consistent over the range 100 ng-1 µg per sample.

The method described above was considered to be both too long and complicated for the routine estimation of DOPEG and MOPEG in human lumbar c.s.f. It was therefore decided to concentrate on the estimation of just one of these noradrenaline metabolites. As MOPEG is the major cerebral deaminated metabolite of noradrenaline (Glowinski, Kopin and Axelrod, 1965) and has been shown to be present in human c.s.f. (Schanberg, Breese, Schildkraut, Gordon and Kopin, 1968) it was decided to devise a method for its extraction from c.s.f., which was suitable for routine use.

Alternative method for the extraction of 3-methoxy-4-hydroxyphenylethyleneglycol

In the estimation of MOPEG in urine, Wilk, Gitlow, Clarke and Paley (1967) used an anion exchange column to separate it from compounds likely to interfere with its estimation. Unlike the carboxylic acids, the phenolic alcohol is not strongly bound onto the

Table 2:3

The percentage recovery of acetyl DOPEG and acetyl MOPEG applied to the origin of a chromatogram of alkali-washed Whatman No. 542 chromatography paper, developed in the organic phase of a modified Bush "C" solvent system (Sharman, 1969) and eluted in deionized distilled water.

Amount of MOPEG placed at origin (ng)	percentage recovery	
	Acetyl MOPEG	Acetyl DOPEG
200	96	93
500	97	96
1000	100	93

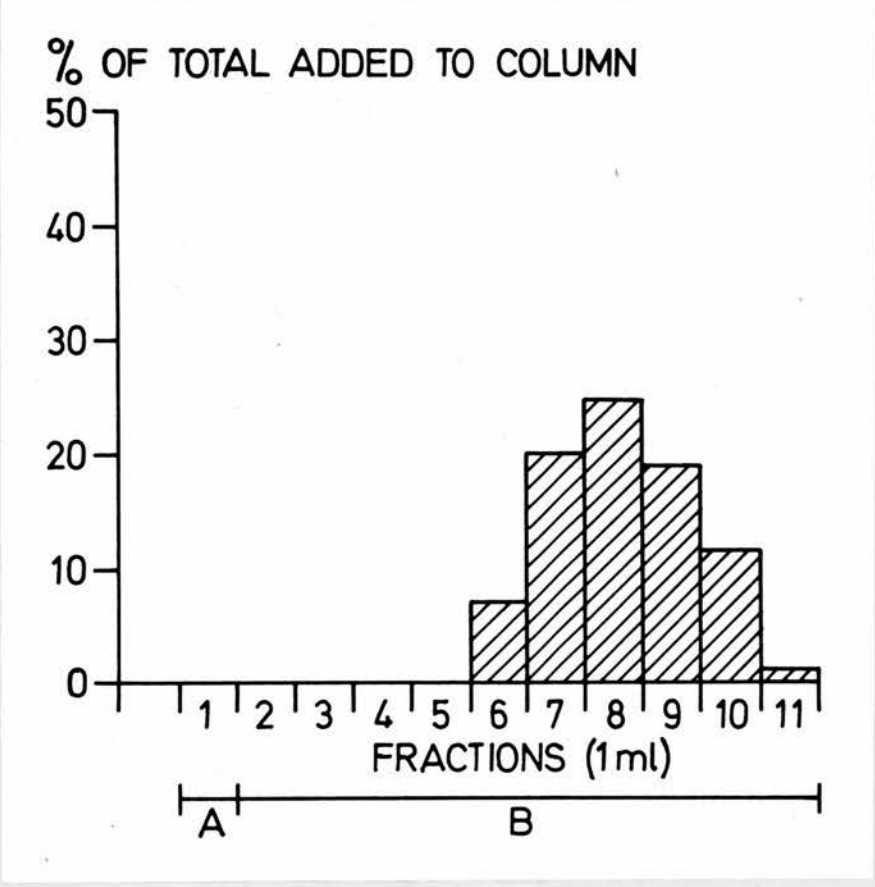


Fig. 2:16

Adsorption and elution of 3-methoxy-4-hydroxyphenylethyleneglycol from a 4 cm x 4 mm column of AG 1 x 4, 200-400 mesh (Bio-Rad) in the chloride form.

A = ml effluent

B = ml eluate

resin, but its flow through the column is retarded, due presumably to a low affinity of the weakly acidic phenolic group for the strongly basic functional groups of the resin, and it can be eluted from the column with water. It was decided to examine this method in an attempt to develop a relatively fast and simple technique for the estimation of MOPEG in c.s.f.

Adsorption and elution of 3-methoxy-4-hydroxyphenylethyleneglycol from the anion exchange column. A solution of 1.0 µg MOPEG in 1 ml of water was placed on the top of a column of AG 1 with 4% cross linkage, 200-400 mesh (Bio-Rad) 4 cm long and 4 mm in diameter. The resin was in the chloride form and, before use, was washed repeatedly by decantation with water to remove the fines. The 1.0 ml sample was run onto the column and the effluent collected in a 1.5 ml polystyrene test tube. The resin was then washed with ten separate 1.0 ml portions of water, each eluate being collected in a 1.5 ml polystyrene test tube. An aliquot (0.5 ml) of each of the eleven samples was taken for the fluorimetric estimation of MOPEG as previously described (page 85).

The pattern of elution of MOPEG from the column is shown in Fig. 2:16. In subsequent experiments the 1.0 ml sample containing MOPEG was run onto the column which was then washed with a further 1.0 ml of water. The MOPEG was then "eluted" from the column in 8.0 ml water which was collected in a 30 ml test tube fitted with a

standard joint and evaporated to dryness under reduced pressure at 40°C. The residue in the tube was dissolved in 0.6 ml water, care being taken that the whole of the inside of the tube was wetted with the solvent. For the fluorimetric estimation of MOPEG as described on page 85, a 0.5 ml portion of the solution was used. The recovery of MOPEG through this method was 75 ± 9 (mean percentage recovery ± standard deviation: 24 estimations) and was consistent within the range 100 ng-1 µg per sample.

This method was both quicker and gave better recoveries than the paper chromatographic method previously described. It was decided to use this procedure as part of the technique for the estimation of MOPEG in c.s.f.

The sulphate ester of 3-methoxy-4-hydroxyphenylethyleneglycol

In the majority of biological tissues so far examined as well as in human lumbar and ventricular c.s.f., MOPEG exists as its sulphate ester (Schanberg, Breese, Schildkraut, Gordon and Kopin, 1968). It was necessary to hydrolyse the conjugate before the alcohol could be extracted and estimated.

Organic sulphate esters in solution can be hydrolysed either by heating them at a low pH value or by the use of a sulphatase enzyme.

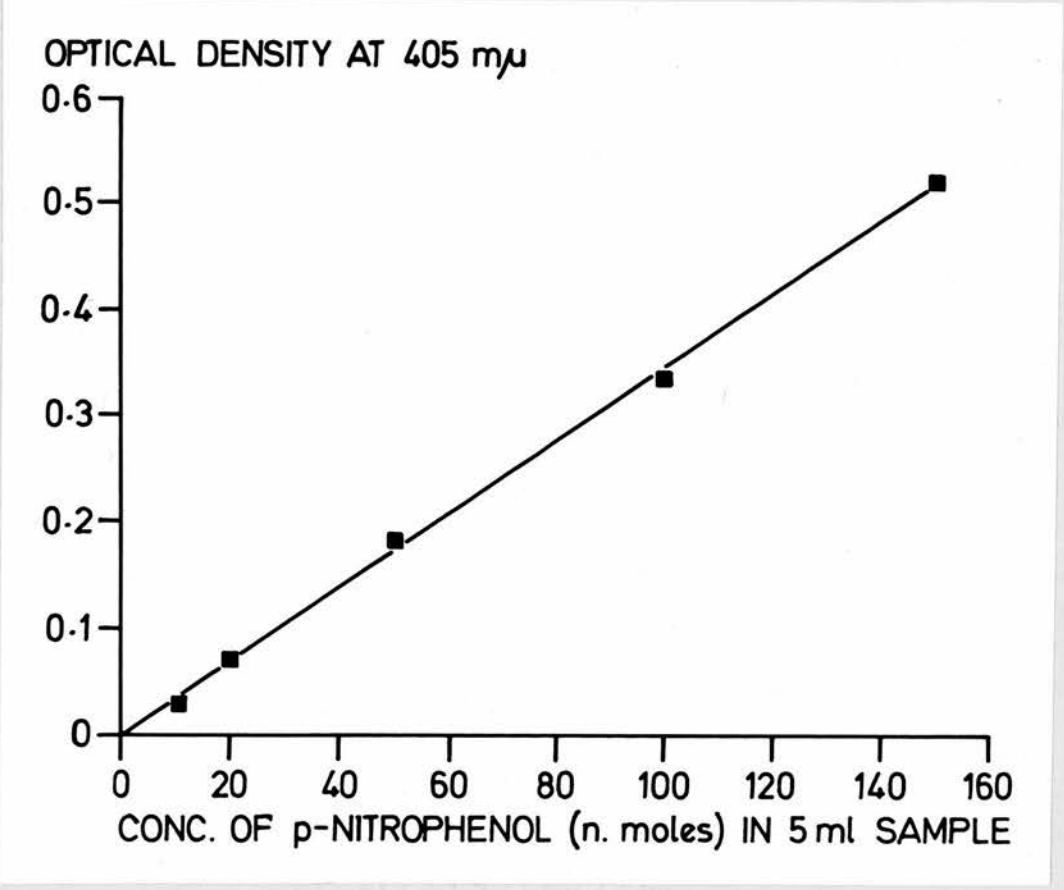


Fig. 2:17

Relation between the optical density at 405 mμ and the concentration (n moles/5 ml sample) of p-nitrophenol after the addition of 0.5 ml of 20% NaOH.

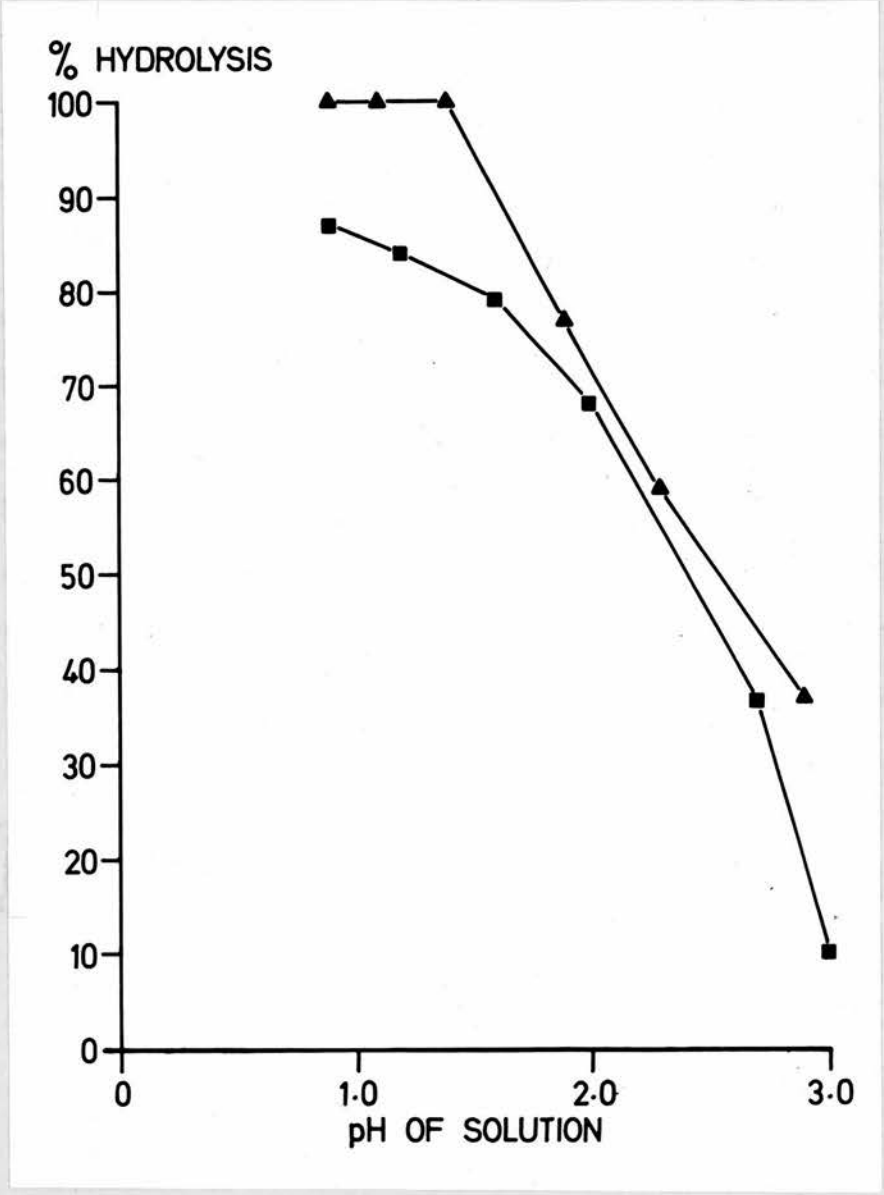


Fig. 2:18

Hydrolysis of p-nitrophenyl sulphate when heated at 100°C for 15 min (■——■) or 30 min (▲——▲) at different pH values.

Acid hydrolysis. In the absence of any MOPEG sulphate another organic sulphate ester, p-nitrophenylsulphate, was used to monitor the degree of hydrolysis obtained when it was heated for different times at different pH values. There was no reason to suppose that the degree of hydrolysis of the latter substance should differ from that of the former under similar conditions.

Solutions of 100 n.moles of p-nitrophenylsulphate in 5 ml of water in 15 ml glass stoppered test tubes were adjusted with 5N and 1N sulphuric acid to a pH within the range 1.0-3.0 and were heated in a boiling waterbath for 15 or 30 mins after which time they were rapidly cooled. The degree of hydrolysis of the sulphate ester was estimated by determination of the liberated p-nitrophenol. To the reaction mixture was added 0.5 ml 20% (w/v) sodium hydroxide and the extinction of the solution at 405 mμ measured in a Unicam SP 500 spectrophotometer. The instrument was set to give 100% transmission with a solution of 100 n.moles p-nitrophenylsulphate to which the sodium hydroxide was added without any prior treatment of the ester solution. In a strongly alkaline medium, p-nitrophenol, but not the sulphate ester, gives a yellow solution, the extinction of which is linearly related to the amount of p-nitrophenol present (Fig. 2:17).

Total hydrolysis of the sulphate ester was only obtained when it was heated for 30 mins at a pH of 1.4 or lower (Fig. 2:18).

The use of acid hydrolysis to obtain free MOPEG from its sulphate

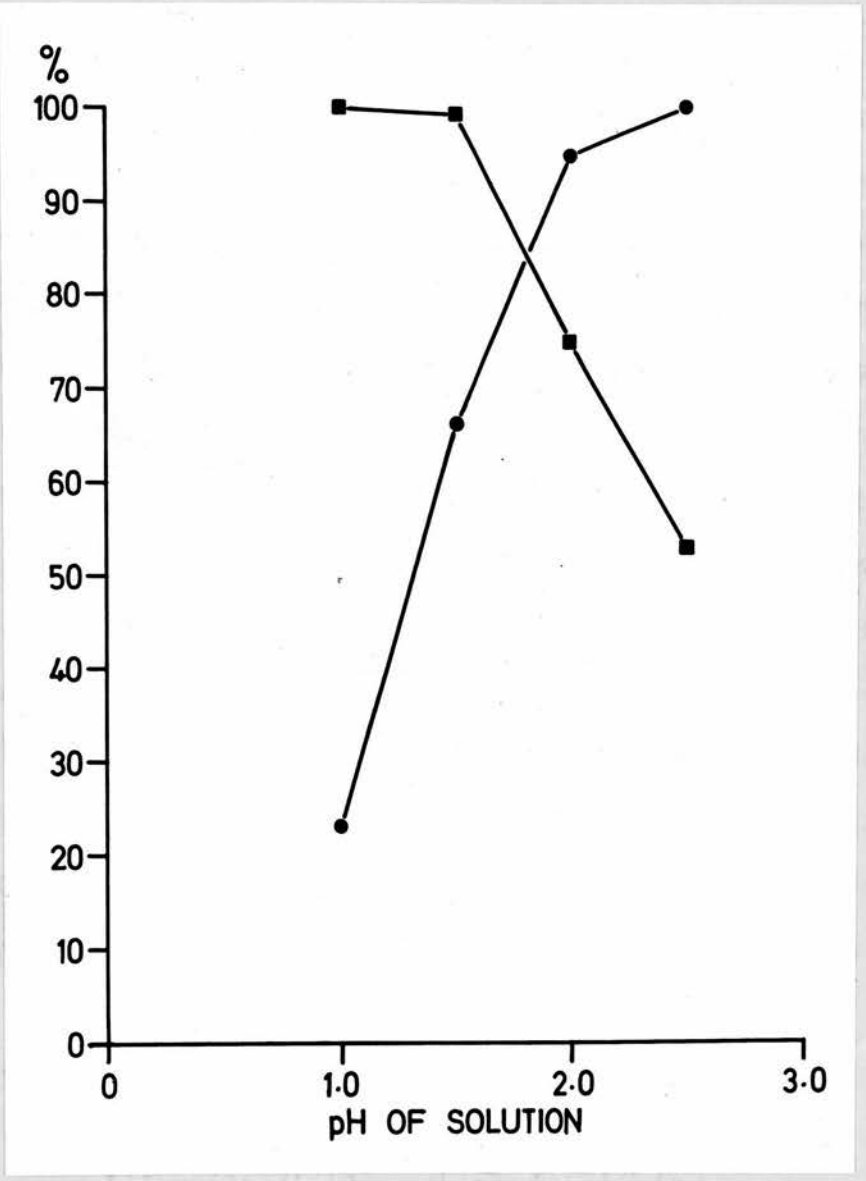


Fig. 2:19

Hydrolysis of p-nitrophenyl sulphate (■——■) and stability of 3-methoxy-4-hydroxyphenylethyleneglycol (●——●) when incubated for 30 min at 100°C in solution at different pH.

ester would necessitate heating the liberated MOPEG at a low pH. It was thus necessary to determine the stability of the glycol under these conditions.

Four solutions each containing 1 µg MOPEG in 5 ml water in 15 ml glass stoppered test tubes were adjusted to pH 1.0, 1.5, 2.0 and 2.5 respectively with 5M and 1M sulphuric acid. For comparison purposes four solutions each containing 100 n.moles p-nitrophenylsulphate in 5 ml water in 15 ml glass stoppered test tubes were adjusted to the same pH values with sulphuric acid. Both series of tubes were heated in a boiling waterbath for 30 mins. After the solutions had been cooled rapidly to room temperature, the degree of hydrolysis of the p-nitrophenylsulphate was estimated as described above. In the other set of tubes MOPEG was estimated by the method of acetylation, extraction with methylene dichloride and chromatography as described on page 103.

When heated for 30 mins at pH 1.5, the highest pH used which gave 100% hydrolysis of the p-nitrophenylsulphate, more than 30% of the MOPEG was destroyed (Fig. 2:19); at pH 2.0, a pH which only destroyed 5% of the MOPEG under the conditions of the experiment, p-nitrophenylsulphate was only 75% hydrolysed (Fig. 2:19).

Enzyme hydrolysis. The enzyme preparation "Helicase" obtained from Industrie Biologique Francais, was a mixed sulphatase and glucuronidase containing 1×10^6 Fishman units of β-glucuronidase

and 1.5×10^7 Roy units of sulphatase per gram, and consisted of a lyophilized preparation of the gastric juices of Helix pomatia. The pH optimum of the sulphatase action was stated by the manufacturers to be pH 5.0.

To monitor the degree of hydrolysis, the sulphate ester of p-nitrophenol was not used as it had been in the experiments on acid hydrolysis because it was thought quite probable that this substance would have a different affinity for the sulphatase than did MOPEG sulphate. Instead tritiated MOPEG sulphate was used as substrate. This was prepared biosynthetically from ^{3}H-normetanephrine as described in the Appendix.

Samples containing approximately 25,000 disintegrations per minute (11.25 nCi) of ^{3}H-MOPEG sulphate in 5 ml water in 30 ml glass stoppered test tubes were adjusted to pH 5.0 by the addition of 1.0 ml 1M acetate buffer pH 5.0 and 0.2 ml of the solution was taken for the determination of tritium by liquid scintillation counting. To each sample was added 20 mg of the enzyme in 0.5 ml water and, in order to prevent the growth of bacteria during incubation, 2 drops of chloroform. Nitrogen was slowly bubbled through the solutions for one minute. The tubes were stoppered and placed in a metabolic shaking waterbath at 37^{o}C for 16 hours. After incubation the contents of the tubes were cooled to room temperature, saturated with sodium chloride and the free ^{3}H-MOPEG extracted from aqueous solution by shaking with 10 ml of ethyl acetate. After centrifugation

at 3,000 g to separate the layers, 8.5 ml of the top organic phase was transferred to a 100 ml round bottomed flask. The extraction was repeated twice with further 10 ml volumes of ethyl acetate, 10 ml of the organic phase from each of the second and third extractions being pooled in the round bottomed flask with the 8.5 ml from the first. Of this 28.5 ml of ethyl acetate 2 ml were taken for the determination of tritium by liquid scintillation counting. The percentage of the original activity extracted into the ethyl acetate was compared with that from an identical sample which had been incubated in the absence of any enzyme and to which the enzyme had been added after salt saturation.

The percentage of the ^{3}H-MOPEG sulphate hydrolysed and extracted into the ethyl acetate was 87 $\pm$ 9 (mean percentage $\pm$ standard deviation: 12 estimations).

In the estimation of MOPEG in c.s.f. the sulphate conjugate of the glycol was hydrolysed by enzyme hydrolysis.

It was found however that the use of the enzyme as supplied by Industrie Biologique Francais gave high background fluorescence in the estimation of MOPEG. An acetone powder of the "Helicase" preparation was therefore, prepared.

One gram of the "Helicase" powder was dissolved in 20 ml of water and was added dropwise to 300 ml of ice cold acetone. The precipitated protein was separated by centrifugation at 1,000 g for 5 mins and the acetone supernatant decanted off. The precipitate

was washed twice with ice cold acetone which had been previously dried over anhydrous sodium sulphate. The acetone powder was dried in a desiccator under vacuum.

This acetone powder of "Helicase" was used in all the estimations of MOPEG in c.s.f. and was found to give a lower background fluorescence than the crude "Helicase" preparation.

THE ESTIMATION OF 3-METHOXY-4-HYDROXYPHENYL-ETHYLENEGLYCOL IN HUMAN CEREBROSPINAL FLUID

Cerebrospinal fluid (c.s.f.). Lumbar c.s.f. was obtained from patients with affective disorders at the Royal Edinburgh Hospital. The c.s.f. was removed and stored as described on page 17. The patients had not received any drugs for at least 24 hours before the removal of the c.s.f.

Estimation of MOPEG in c.s.f. A sample of lumbar c.s.f. (5 ml) was placed in a 15 ml glass stoppered test tube and its pH adjusted to 5.0 by the addition of 1.0 ml 1M acetate buffer pH 5.0. The sulphatase-containing enzyme preparation "Helicase" (20 mg) in 0.5 ml water was added to this solution and in order to prevent the growth of bacteria during incubation, 2 drops of chloroform were introduced into each tube. After slowly bubbling nitrogen through the solutions for one minute the tubes were stoppered and placed in a metabolic shaking waterbath at 37°C for 16 hours. After incubation the contents of the tubes were cooled to room temperature and the

protein in them precipitated by the addition of 0.2 ml 40% w/v zinc sulphate followed by 0.15 ml of 20% w/v sodium hydroxide. The tubes were centrifuged at 3,000 g for 5 mins and the supernatant fluid transferred to a 30 ml glass stoppered test tube. The precipitate was resuspended in 1.0 ml of water and the tubes were again centrifuged at 3,000 g for 5 mins. The supernatant fluid was pooled with that from the first centrifugation. The pooled extracts were saturated with sodium chloride and the free MOPEG extracted from aqueous solution by shaking it for 5 mins with 10 ml of freshly redistilled ethyl acetate. After centrifugation at 3,000 g for 5 mins to separate the layers, 8.5 ml of the top organic phase was transferred to a 100 ml round bottomed flask. The extraction was repeated with a further 10 ml of ethyl acetate, 10 ml of the organic phase being pooled in the round bottomed flask with the 8.5 ml from the first extraction. The pooled ethyl acetate extracts were evaporated to dryness at room temperature under reduced pressure.

The residue in the flask was dissolved in 1.0 ml water and the solution was run onto the top of a 4 cm long by 4 mm in diameter column of AG 1 x 4 200-400 mesh (Bio-Rad) in the chloride form. The flask was washed with a further 1.0 ml of water which was also added to the top of the column.

Any MOPEG was then eluted from the column in 8.0 ml of water which were collected in a 30 ml glass stoppered test tube and evaporated to dryness under reduced pressure at 40^{o}C. The residue

in the tube was dissolved by washing the sides of the tube with 0.6 ml of water, care being taken to wet the whole of the inside of the tube with solvent. The MOPEG in 0.5 ml of the solution was estimated fluorimetrically.

The fluorescence in the final extract due to the reagents was determined by processing 5.0 ml of deionised distilled water through the method. Recoveries of MOPEG of between 100 and 500 ng in 5.0 ml of water or, in certain cases, where 10 ml of the original c.s.f. sample was available, from c.s.f., were also processed through the method.

Results

Recoveries. The recovery of 100, 200 or 500 ng of MOPEG when added to 5 ml of water or 5 ml of c.s.f. prior to incubation with the sulphatase preparation "Helicase" and extraction into ethyl acetate as described on page was 50 $\pm$ 5 (mean percentage recovery $\pm$ standard deviation: 12 estimations). The recovery of MOPEG was the same from c.s.f. as from water and was more consistent within a series of estimations than it was between series.

Sensitivity of method. The method as described could estimate down to 50 ng of MOPEG in 5 ml of deionized distilled water. In c.s.f., however, the background fluorescence was greater and the limit of sensitivity was about 100 ng in a 5 ml sample.

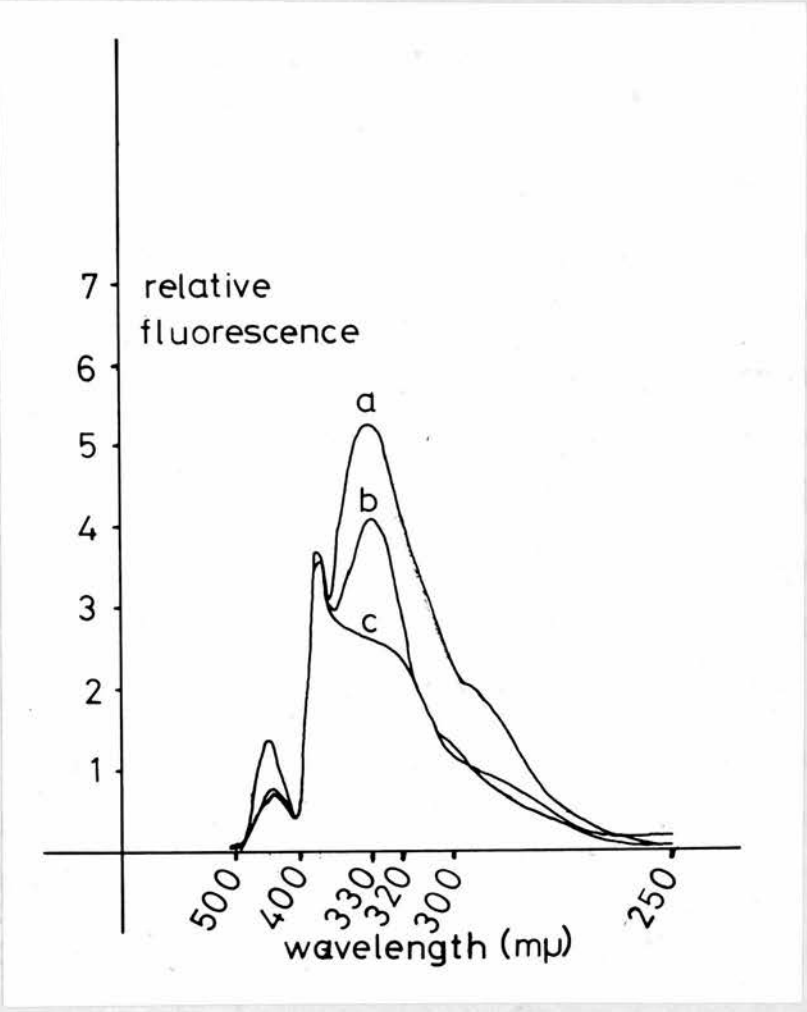


Fig. 2:20

The figure shows an activation spectrum of (a) 5 ml of cerebrospinal fluid (b) 100 ng of 3-methoxy-4-hydroxyphenylethyleneglycol in 5 ml of water and (c) 5 ml of water, processed through the method for the estimation of 3-methoxy-4-hydroxyphenylethyleneglycol in c.s.f.

The activation spectrum was recorded from 250 mμ to 500 mμ with the fluorescence monochromator set at 430 mμ.

Characterisation of the substance estimated in c.s.f. The substance which was present in the c.s.f. after it had been incubated for 16 hours at 37°C with a sulphatase preparation "Helicase", was similar to MOPEG in several respects. It was extractable into ethyl acetate from a salt saturated aqueous solution at pH 5.0 and its flow through the anion exchange AG 1 x 4 column was retarded in a way similar to that of MOPEG.

Furthermore the fluorescence characteristics of the fluorophor formed from the substance present in the final extract from c.s.f. were the same as those of authentic MOPEG taken through the method (Fig. 2:20).

Ideally the substance estimated in the c.s.f. should have been further characterised by paper or thin layer chromatography in order to determine whether it had the same chromatographic mobility as authentic MOPEG. This was not attempted because of the difficulty in obtaining sufficient c.s.f. for this purpose.

3-methoxy-4-hydroxyphenylethyleneglycol in human lumbar c.s.f. The method was applied to the estimation of MOPEG in human lumbar c.s.f.

The c.s.f. (10 ml) was withdrawn from patients with various neurological disorders and was stored as described on page 17. An aliquot of the c.s.f. (5 ml) was used for the estimation of MOPEG and the remainder used for the estimation of 3-methoxy-4-hydroxyphenyl-acetic acid (homovanillic acid, HVA) and 5-hydroxyindol-3-ylacetic

acid (5-HIAA). The results are shown in Table 2:4)

DISCUSSION

The biogenic amine, dopamine, is catabolised within the central nervous system predominantly to the phenolic acids 3,4dihydroxyphenylacetic acid (DOPAC) and 3-methoxy-4-hydroxyphenylacetic acid (homovanillic acid, HVA) while the major metabolites of its β-hydroxylated derivative, noradrenaline, are the neutral phenolic glycols, 3,4dihydroxyphenylethyleneglycol (DOPEG) and 3-methoxy-4-hydroxyphenylethyleneglycol (MOPEG) (Rutledge and Jonason, 1967; Breese, Chase and Kopin, 1968; Breese, Chase and Kopin, 1969).

Both DOPAC and HVA are present in cerebrospinal fluid (c.s.f.) (Andén, Roos and Werdinius, 1963b; Ashcroft, Crawford, Dow and Guldberg, 1968) and estimation of their concentrations has been used by many workers to indicate changes in the turnover or metabolism of dopamine within the central nervous system brought about by neurological diseases or by drug treatment. That changes in the concentrations of these acid catabolites can reflect changes in the metabolism of the parent amine has been discussed by Guldberg, Ashcroft, Turner and Hanieh (1969) and also in this thesis (page 65).

There are as yet few data concerning the glycol metabolites of noradrenaline, DOPEG and MOPEG, in c.s.f. The presence of MOPEG, as a conjugate, probably the sulphate ester, in human lumbar c.s.f. was demonstrated by Schanberg, Breese, Shildkraut, Gordon and Kopin

Table 2:4

The concentrations (ng/ml) of 3-methoxy-4-hydroxyphenylethyleneglycol (MOPEG), 3-methoxy-4-hydroxyphenylacetic acid (HVA) and 5-hydroxyindol-3-ylacetic acid (5-HIAA) in the lumbar c.s.f. of patients with various neurological disorders.

Patient	Diagnosis	Age	MOPEG	HVA	5-HIAA
1	Depressive illness	45	74	9	12
2	Depressive illness	51	24	17	10
3	Depressive illness	51	21	11	7
4	Depressive illness	57	17	12	8
5	Manic-depressive (depressed)	62	30	7	24
6	Manic-depressive (depressed)	60	21	23	15
7	Manic-depressive (manic)	64	170	27	26
8	Manic-depressive (manic)	66	52	20	10
9	Manic-depressive (manic)	62	41	36	19
10	Manic-depressive (manic)	52	33	13	9
11	Manic-depressive (manic)	62	33	43	12
12	Post-traumatic parkinsonism	51	40	14	27
13	Parkinsonism on 3 g L-DOPA/day	61	51	291	13

(1968), who estimated its concentration to be between 50 and 200 ng/ml. There appears to have been no report on the presence of DOPEG.

The more extensive study of the concentrations of the noradrenaline metabolites in c.s.f. has been prevented by the absence of a method which was both sensitive yet simple enough to be used for routine estimations. The estimation of MOPEG by converting it to vanillin and estimating the vanillin by its absorption at 360 mμ as described by Ruthven and Sandler (1965) for the estimation of this phenolic glycol in urine, is not sensitive enough to allow its application to c.s.f. analysis. Gas chromatographic methods described by Wilk, Gitlow, Clarke and Paley (1967) and Sharman (1969) for the estimation of MOPEG and DOPEG are very much more sensitive, and that of Wilk et al., (1967) was used by Schanberg, Breese, Schildkraut, Gordon and Kopin (1968) for the estimation of MOPEG in human lumbar c.s.f. and ventricular c.s.f. Both these gas chromatographic methods employ electron capture detection. Because of the high risk of contamination and long recovery time of the electron capture head, together with the unduly high sensitivity and consequent likely proneness to errors by the presence of contaminants, they are not altogether suitable, in their present state of development, for routine analysis.

It seemed probable, prior to the advent of the gas chromatographic methods mentioned above, that methods of sufficient sensitivity and specificity for the routine estimation of the glycols in low concentrations would be dependent on a fluorimetric method of assay.

It was with this in mind that the development of the fluorimetric techniques described in this section of the thesis was embarked upon.

DOPEG was found to give a fluorophor on reaction with ethylene diamine under the conditions used by Rosengren (1960) for the estimation of DOPAC. The fluorescence intensity of the derivative was such that DOPEG could be estimated, in solution in water, in a concentration as low as 4 ng/ml. This method is not specific for DOPEG as ethylene diamine will condense with many compounds containing a catechol grouping (Weil-Malherbe and Bone, 1952; Weil-Malherbe and Bone, 1957; Rosengren, 1960; Ashcroft, Crawford, Dow and Guldberg, 1968) to give a product with similar fluorescence characteristics. Specificity must therefore depend on preliminary adequate separative procedures as discussed later.

The first attempt to develop a fluorimetric method for the estimation of MOPEG was based on a method described briefly by Goldenberg and White (1962) for the estimation of the major peripheral metabolite of noradrenaline 3-methoxy-4-hydroxymandelic acid (vanillin mandelic acid, VMA). The procedure involved the oxidation of VMA to vanillin by reaction with periodate, followed by the condensation of the aldehyde group of the vanillin with thiosemicarbazide to give a fluorescent thiosemicarbazone. This method should have been directly applicable to the estimation of MOPEG as it is also oxidized by periodate to vanillin (Ruthven and Sandler, 1965). It was found, however, that the method, as

described, was unsuitable for the estimation of nanogram quantities of VMA or MOPEG. With modification of the procedure it was found that the condensation of nanogram quantities of vanillin with thiosemicarbazide could be achieved to give a product with fluorescence proportional to the concentration of vanillin in the original solution. Unfortunately no suitable reaction conditions were found to give consistent conversion of nanogram amounts of VMA or MOPEG to vanillin in the first stage of the proposed procedure.

The method used eventually for the estimation of MOPEG was a modification of that described by Sharman (1963) for the estimation of HVA. The procedure involved the oxidation of the MOPEG, with colloidal ferric hydroxide and the precipitation of the ferric ions with sodium hydroxide. The fluorophor thus produced had a high fluorescence intensity enabling the estimation of MOPEG, in solution in water, in concentrations as low as 25 ng/ml. This reaction was not specific for MOPEG as it also produced fluorophors with the same activation and fluorescence characteristics from other O-methylated catechol amine derivatives, (Table 2:2). Of the compounds tested, 3-methoxytyramine, HVA and VMA all gave a fluorophor with a greater fluorescence per unit weight than did MOPEG. A side chain with at least two carbon atoms seemed to be necessary as no fluorescence was obtained from 3-methoxy-4-hydroxybenzaldehyde (vanillin) when it was taken through the fluorophor-producing reaction (Table 2:2). The method did not however produce a fluorophor from catechol-

containing compounds.

As the fluorimetric procedures for the estimation of DOPEG and MOPEG are not specific to these compounds, specificity must be achieved by adequate preliminary separative techniques.

Two methods for the partial purification of the phenolic glycol metabolites of noradrenaline have been investigated. The first involved the acetylation of MOPEG and DOPEG, the extraction of their acetyl derivatives into methylene dichloride and their separation by descending paper chromatography an alkali-washed Whatman No 542 chromatographic paper. In model experiments starting with aqueous solutions of 100 ng-1µg of the compounds this procedure gave a recovery of 60 $\pm$ 3% (mean recovery $\pm$ standard deviation: 4 experiments) for DOPEG and 63 $\pm$ 4% (mean recovery $\pm$ standard deviation: 16 experiments) for MOPEG. It had the advantage that it separated the two phenolic glycols thus eliminating the necessity to divide the sample into two portions; one for the estimation of DOPEG, the other for MOPEG. The phenolic glycols were present in eluates from the chromatogram as their acetylated derivatives. This was of no disadvantage in the estimation of DOPEG because, under the reaction conditions, ethylene diamine produces a fluorophor from the acetylated derivative as readily as from the parent glycol.

Oxidation of acetyl MOPEG with ferric chloride does not produce a fluorophor and so this compound had to be hydrolysed prior to estimation. This was done in alkali and necessitated the

adjustment of the reaction solution to between pH 6.5 and 7.5 before the addition of the ferric chloride reagent.

This separation technique, including the time required for preparation of the paper for chromatography, was considered to be too time consuming to be really suitable as a routine method for the estimation of these glycols in human lumbar c.s.f. and a simpler procedure was therefore sought.

In the development of an alternative procedure, attention was concentrated on the separation and estimation of MOPEG, the major metabolite of noradrenaline in the central nervous system (Glowinski, Kopin and Axelrod, 1965). The separation technique was a modification of that described by Wilk et al., (1967) and involved the extraction of MOPEG from aqueous solution into ethyl acetate and its further purification on an anion exchange column. The recovery of between 100 ng and 1 µg of MOPEG was 75 ± 9%(mean recovery ± standard deviation: 24 estimations). This procedure was both simple and quick and was used for the extraction, prior to estimation, of MOPEG in human lumbar c.s.f. Theoretically, the method should also be suitable for the extraction of DOPEG.

In human c.s.f. MOPEG exists as a conjugate, believed to be its sulphate ester (Schanberg, Breese, Schildkraut, Gordon and Kopin, 1968), and it was necessary to hydrolyse this conjugate before the MOPEG could be extracted into an organic solvent.

Two methods of hydrolysis of the MOPEG conjugate were studied,

hydrolysis with acid and hydrolysis with a sulphatase-containing preparation. It was found that with acid hydrolysis, the conditions of pH and temperature which were required to hydrolyse a typical phenolic sulphate ester (p-nitrophenysulphate) destroyed free MOPEG. This observation is consistent with the findings of Wilk et al., (1967) that acid hydrolysis of urine yielded only 8% of the MOPEG which could be liberated from the urinary conjugate by an enzymatic method.

In order to monitor the hydrolysis of the MOPEG conjugate when it was incubated with the sulphatase-containing preparation "Helicase", experiments were carried out using, as substrate, labelled MOPEG conjugate which had been prepared biosynthetically from tritiated normetanephrine. After incubation with "Helicase", for 16 hours at 37°C, 87 ± 9% (mean percentage ± standard deviation: 12 experiments) of the radioactivity in the original solution could be extracted into ethyl acetate. This compares with less than 2% extractable when the conjugate was incubated in the absence of the sulphatase-containing preparation. The radioactivity present in the ethyl acetate extract of the hydrolysate had the same chromatographic mobility as authentic MOPEG (page 188). It thus seems that there was little or no destruction of the free MOPEG liberated during the period of incubation with the enzyme preparation. This method of hydrolysis was used for the estimation of MOPEG in human lumbar c.s.f.

The evidence that the conjugate of MOPEG is the sulphate ester is not conclusive and is based on the observation that it will only give a substance with the same chromatographic mobility as authentic MOPEG when it is incubated with the sulphatase-containing preparation "Glusulase" (Endo products N.Y.) and not when incubated with pure glucuronidase or phosphatase-containing preparations (Schanberg, Schildkraut, Breese and Kopin, 1968). "Glusulase", which is similar to "Helicase", is a crude preparation of the gastric juice of Helix pomatia and contains many enzyme activities. It is thus possible that it is not the sulphatase activity but some other, unspecified, enzyme activity which is responsible for the hydrolysis of the MOPEG conjugate.

The analytical procedure, as detailed for the estimation of MOPEG in 5 ml of human lumbar c.s.f., allowed the estimation, in model experiments, of down to 50 ng in 5 ml of water. In the extract from 5 ml of c.s.f. however, the nonspecific "blank" fluorescence was greater and the limit of estimation was about 100 ng in the 5 ml sample of c.s.f. (20 ng/ml). Although not as high as might be considered desirable, this level of sensitivity has proved adequate for the estimation of MOPEG in the several c.s.f. samples analysed.

Of the 13 estimations of MOPEG in human lumbar c.s.f. (Table 2:4) only four lie within the range of 50-200 ng/ml quoted by Schanberg, Breese, Schildkraut, Gordon and Kopin (1968). However, it should be pointed out that in the present study the c.s.f. was from patients

with neurological disorders and not from "control"subjects.

The estimations of MOPEG have been carried out on samples from an insufficient number of patients with the same neurological disease to enable any definite conclusion to be drawn regarding the relation of the MOPEG concentrations to the clinical state. However, it is perhaps worthy of some comment that the concentration of MOPEG in the lumbar c.s.f. of patients who had been rated depressed (No.1-6: Table 2:4) tended to be lower than in the c.s.f. of patients rated manic (No.7-11: Table 2:4). This difference however was found not to be significant ($p < 0.2$). If one may be allowed to speculate in the face of an admitted scarcity of evidence, two points suggest themselves by way of explanation of the lack of difference in the concentration of MOPEG in the c.s.f. of depressed and manic patients. Perhaps the MOPEG c.s.f. levels are associated with some more subtle differentiation in symptomatology or perhaps any change in noradrenaline metabolism is being inadequately monitored by estimation of total MOPEG (free and combined) only. Extension of the method to include separate estimations in the c.s.f. of free and conjugated MOPEG and of free and combined DOPEG, should these be present, might be necessary to detect, and follow adequately, changes in cerebral noradrenaline metabolism in various mental diseases.

SECTION 3

The accumulation of ^{14}C-5-hydroxyindol-3-ylacetic acid by the rabbit choroid plexus *in vitro*.

INTRODUCTION

Transport system for the removal of many substances from the cerebrospinal fluid (c.s.f.) have been shown to exist. In 1961, Pappenheimer, Heissey and Jordan studied the removal of diodrast and phenolsulphonphthalein from the cerebral ventricles of the goat. These workers used a technique of 'open-ended' perfusion where the substance, in artificial c.s.f. was pumped at a known and constant rate through a cannula into a lateral ventricle. The artificial c.s.f. flowed through the ventricular system and was collected from an outflow cannula situated in the cisterna magna. Both diodrast and phenolsulphonphthalein were found to be rapidly cleared from the perfusate by a saturable mechanism. By altering the sites of the inflow and outflow cannulae it was possible to show that the removal mechanism was situated in the fourth ventricle.

This technique of ventricular perfusion was used by Davson, Kleeman and Levin (1962) to study the removal of p-aminohippuric acid (PAH) from the ventricles of the cat and by Pollay and Davson (1963) for the removal of PAH, thiocyanate and iodide from rabbit c.s.f. These substances were rapidly cleared from the ventricles by a mechanism which was saturable and the presence of one of the anions in the perfusate inhibited the removal of another. There proved to be a species difference in the sites of this removal.

In the cat PAH was only transported out of the c.s.f. in the region of the lateral ventricles (Davson et al., 1962) whilst in the rabbit it was removed from the perfusate in all the ventricles (Pollay and Davson, 1963). More recently Ashcroft, Dow and Moir (1968) showed in the dog the existence of an active transport system, located in the fourth ventricle, for the removal of 5-hydroxyindol-3-ylacetic acid (5-HIAA) and 3-methoxy-4-hydroxyphenylacetic acid (homovanillic acid, HVA).

Cationic substances such as hexamethonium, decamethonium and N'-methylnicotinamide are also transported out of the ventricular system (Schanker, Prockop, Schou and Sisodia, 1962). There was competition between these bases for the removal mechanism.

The structures implicated in this transport process are the highly vascular choroid plexuses. These are situated within the ventricles , have a large surface to volume ratio and have epithelial cells that are analogous in structure to those in the proximal tubule of the kidney (Davson, 1967). Evidence to support the idea of the involvement of the choroid plexuses in the mechanism for the transfer from c.s.f. to blood has been obtained by in vitro studies of their ability to accumulate certain substances. The technique used in such studies on the isolated choroid plexuses was essentially similar to that used by Cross and Taggart (1950) and Despopoulous and Weisbach (1957) for the study of the uptake of PAH and 5-HIAA by kidney slices.

Welch (1962a,b) demonstrated the accumulation of iodide and thiocyanate by the choroid plexuses of the rabbit. The accumulation of iodide, which was greater in the fourth ventricular choroid plexus than in those from the lateral ventricles was inhibited by the metabolic inhibitors, fluoride, cyanide, malonate, iodoacetate, 2,4-dinitrophenol and fluoroacetate as well as by cardiac glycosides. Both iodide and thiocyanate appeared to be transported by the same system as the presence of one in the incubation medium inhibited the accumulation of the other. Robinson, Cutler, Lorenzo and Barlow (1968) confirmed the capacity of the rabbit choroid plexuses to accumulate iodide and also showed that they were capable of the uptake of sulphate and thiosulphate. The transport of these latter anions was by a separate mechanism as they did not interfere with the accumulation of the iodide ion.

The isolated choroid plexus is also capable of accumulating amino acids. Lorenzo and Cutler (1969) showed that the isolated choroid plexus of the cat could accumulate the non-metabolizable amino acid, α-amino-isobutyric acid. The extent of the accumulation was very much smaller than observed for the simple anions. There was competition for uptake with other amino acids.

Cationic compounds are also accumulated by the isolated choroid plexus. Tochino and Schanker (1965a,b) used the tissue from rabbits to study the uptake of 5-hydroxytryptamine, noradrenaline, decamethonium, hexamethonium and

N'-methylnicotinamide. These authors showed that the accumulation of these substances could take place against a concentration gradient and that the lateral ventricular choroid plexuses contained a more efficient accumulation mechanism than that from the fourth ventricle. The uptake of any one of these cations was inhibited by the presence of any of the others or of metabolic inhibitors in the incubating medium. The rabbit choroid plexuses have also been shown to be capable of accumulating morphine and morphine derivatives (Takemori and Stenwick, 1966; Hug, 1967).

Unionized compounds such as carbohydrates are also accumulated by the isolated choroid plexus (Csaky and Rigor, 1964). These authors showed that the isolated choroid plexuses of the dog could accumulate glucose, by a mechanism which was dependent on metabolic energy, to give a concentration in the tissue up to twenty times that in the medium.

There is thus evidence that both anionic and cationic compounds can be transported out of the c.s.f. Evidence in support of a role for the choroid plexuses in this removal is afforded by the ability of the cells of the isolated tissue to accumulate such ions by energy dependent processes.

The experimental work described below represents an extension of investigations in this laboratory by Ashcroft, Dow and Moir (1968) who studied the kinetics of the removal of 5-HIAA from a perfusate of the cerebral ventricles in the conscious dog. They

found that in addition to 'Bulk flow', part of this removal could be accounted for by a saturable mechanism located in the region of the fourth ventricle. From the literature it seemed probable that the choroid plexus might be responsible for this removal and the experiments to be described were undertaken to investigate whether the isolated choroid plexus is able to accumulate 5-HIAA by a saturable, energy dependent process.

MATERIALS

Homovanillic acid was obtained from Calbiochem and probenecid from Merck, Sharpe and Dohme. Other chemical reagents were of at least 'Analar' (British Drug Houses) standard of purity. Deionised distilled water was used throughout.

Inulin - ^{14}C-carboxylic acid, 1.35 μCi/mg (approx 6.75 mCi/m. mole), was obtained from the Radio-chemical Centre (Amersham).

Radioactive 5-hydroxyindol-3-ylacetic acid ^{14}C-5-HIAA, 25 mCi/m. mole, was obtained as a solution in methanol from the New England Nuclear Corporation and before use was purified as follows. The contents of one sealed ampoule (0.05 mCi) were made up to 3.0 ml with water in a 15 ml glass stoppered test tube. The solution was saturated with sodium chloride, acidified with one drop of conc. HCl and the ^{14}C-5-HIAA extracted by shaking the solution for 5 minutes with 6 ml ether. The two phases were allowed to

separate and the upper organic layer was transferred to a 30 ml glass stoppered test tube. The extraction was repeated twice with further 6 ml volumes of ether (the ether used in the extractions was purified immediately before use by shaking it with an equal volume of a saturated solution of ferrous sulphate, followed by distillation). The ^{14}C-5-HIAA was returned to aqueous solution by shaking the combined ether extracts with 2.0 ml 0.1M phosphate buffer pH 7.4 and, after separation of the two phases, the bottom aqueous layer was transferred to a glass vial. The residual ether was removed from the phosphate buffer extract by blowing a stream of nitrogen over it until no smell of ether was detectable. The activity of this solution was estimated on a Packard Tri-carb liquid scintillation counter (see page 150). The concentration of 5-HIAA was calculated from the disintegrations per minute and the specific activity of the particular batch of ^{14}C-5-HIAA as quoted by the manufacturers.

Scintillant for liquid scintillation counting The scintillant consisted of a solution of 10.63 g 2,5-diphenyloxazole (PPO) and 0.28 g 1,4-bis-(2-(4-methyl-5-phenyloxazolyl))-benzene (dimethyl POPOP) in 2.5 litres of toluene.

METHODS

Removal of the choroid plexuses

New Zealand white rabbits of either sex were killed by the intravenous injection of 50 ml of air and bled out from severed blood vessels in the neck. The top of the skull was rapidly cut off and the brain removed onto a glass plate. With a pair of forceps it was split down the midline through the corpus callosum to the floor of the third ventricle. The lateral ventricles were opened and their choroid plexuses cut at their point of attachment at the junction of the lateral and third ventricles. The fourth ventricular choroid plexus was removed by gently easing it from the roof and sides of the ventricular space. It was, however, much more difficult to dissect than the plexuses from the lateral ventricles and preparations obtained were not always suitable for use.

Oxygen consumption

In order to establish that the method used for the dissection of the choroid plexuses from the ventricles of the rabbit gave viable preparations, the oxygen consumption of the tissue was measured over a period of 45 min.

The choroid plexuses from the lateral, third and fourth ventricles from five animals were weighed on a torsion balance and placed in 1.0 ml Krebs' - Ringer phosphate solution (Cremer, 1957) in five separate Warburg flasks; each flask

containing the combined choroid plexuses from one animal. Carbon dioxide formed on incubation of the tissue was absorbed by 0.2 ml 6N sodium hydroxide in the centre well of the flask into which had been placed a pleated 'fan' of Whatman No. 1 filter paper to increase the surface area of the absorbing solution. The side arm of two of the flasks contained 0.01 ml 10^{-2} M 2,4-dinitrophenol. Two control flasks with medium and sodium hydroxide but without any tissue, and an empty flask, which served as a thermobarometer, were also included. After gassing the flasks and manometers with oxygen for 10 mins and equilibrating them at 37°C for a further 15 mins, they were shut to the atmosphere and the two containing the 2,4-dinitrophenol were tilted to allow the incubating solution to mix with the contents of the sidearm (the final concentration of 2,4-dinitrophenol in the incubating medium was 10^{-4}M). The manometers were read at zero time and at 5 mins intervals, up to and including 45 mins.

Incubation of the choroid plexuses

In subsequent experiments the choroid plexuses were incubated in the physiological salt solution described by Welch (1962a), the composition of which is given in Table 3:1. To obtain a pH of 7.4 the solution was equilibrated with 95% O_2: 5% CO_2 for 20 mins. An aliquot of the salt solution, 1.0 ml, was introduced into a glass vial with an internal diameter of 2 cm, and to it was added the substance under test along with any inhibitor or other

TABLE 3:1

Physiological Salt Solution (Welch 1962a)

	Concentration (g/l) of anhydrous salts
Sodium chloride	8.0
Potassium chloride	0.2
Calcium chloride	0.1
Magnesium chloride	0.1
Sodium bicarbonate	1.0
Sodium dihydrogen orthophosphate	0.05
Glucose	1.0

The solution was brought to pH 7.4 by equilibrating with 95% O_2: 5% CO_2

compound. A choroid plexus was then placed in the vial and the atmosphere above the incubation fluid changed to 95% O_2: 5% CO_2 by passing a stream of this gas mixture into the vial for 15 secs. The vial was sealed with a clip-on plastic top and incubated in a metabolic shaking waterbath.

Uptake of ^{131}I-iodide by the isolated choroid plexus

The ability of the lateral ventricular choroid plexus to accumulate iodide was tested as this ion had already been shown to be actively taken up by this tissue. (Welch, 1962a; Robinson et al., 1968).

The lateral ventricular choroid plexuses were removed as previously described, and incubated at 30°C for one hour in the presence of 0.5 or 1.0 µCi/ml carrier-free ^{131}I-iodide. After incubation the tissue was blotted gently on Whatman No. 1 filter paper, weighed on a torsion balance and placed in a glass vial. The ^{131}I-iodide in the tissue and also in 10 µl of the medium was estimated in a well-type γ-scintillation counter. The incubation was also carried out in the presence of 2,4-dinitrophenol.

Inulin space of the rabbit choroid plexus

Estimation of the quantity of a radioactive compound in a choroid plexus after it has been incubated in a solution containing that compound will give the total amount within the cells of the tissue as well as in the extracellular fluid. In order to determine the intracellular concentration the volume of the extracellular space must be known. This can be achieved by

incubating the choroid plexus with a substance which will not pass into the cells but which will enter the extracellular space. Such a compound is inulin and the 'space' with which it comes into equilibrium is the 'inulin space'.

The inulin space of the lateral and fourth ventricular choroid plexuses of the rabbit was estimated by incubating them at 30°C in 1.0 ml of the physiological salt solution containing 82.2 µg (0.125 µCi) inulin - ^{14}C-carboxylic acid, under an atmosphere of 95% O_2: 5% CO_2 for times ranging from 10 to 80 mins. The plexuses were then removed from the incubation medium, lightly blotted on Whatman No. 1 filter paper, weighed on a torsion balance and placed in the vial of a liquid scintillation counter. An aliquot of the incubation medium, 0.1 ml, was placed in a separate vial (see page 150).

The accumulation of ^{14}C-5-hydroxyindol-3-ylacetic acid by the rabbit choroid plexus

Whole choroid plexuses from lateral or fourth ventricles were incubated at 30°C or 6°C in 1.0 ml of the physiological salt solution containing between 50 ng and 5.0 µg ^{14}C-5-hydroxyindol-3-ylacetic acid (^{14}C-5-HIAA) for varying lengths of time under an atmosphere of 95% O_2: 5% CO_2. Any inhibitor or other substance to be added to the incubation medium was introduced in a volume of 0.01 ml prior to placing the choroid plexus in the incubation flask. After incubation, the choroid plexus was removed from the flask,

blotted on Whatman No. 1 filter paper and, after weighing on a torsion balance, placed in a vial for liquid scintillation counting. A portion, 0.1 ml, of the incubation medium was placed in a separate vial for the estimation of the ^{14}C-labelled 5-HIAA, (see page 150).

Incubations involving the lateral choroid plexuses were all done in duplicate and in no case were the duplicates from tissue of the same animal. As there is only one fourth ventricular choroid plexus per animal incubations involving this tissue were not carried out in duplicate.

Liquid scintillation counting

Liquid scintillation counting was used to estimate ^{14}C-labelled compounds in the tissue and medium. Tissue, prior to counting, was digested overnight at room temperature in 0.2 ml 2.5M KOH in the scintillation vials. This gave a clear, homogeneous solution with a slight brown colouration. These aqueous samples, however, were not miscible with the toluene scintillant and therefore 4 ml of ethanol/methanol (3:1 v/v) was added to each vial before the 10 ml of scintillant. By this method up to 0.3 ml of an aqueous sample could be accommodated by the 10 ml of toluene scintillant.

The radioactivity of the samples was estimated in a Packard Tri-Carb Scintillation Counter. Each sample was counted over a period of 100 mins or until such time as 10,000 counts had been registered. The latter method yields a count with a statistical

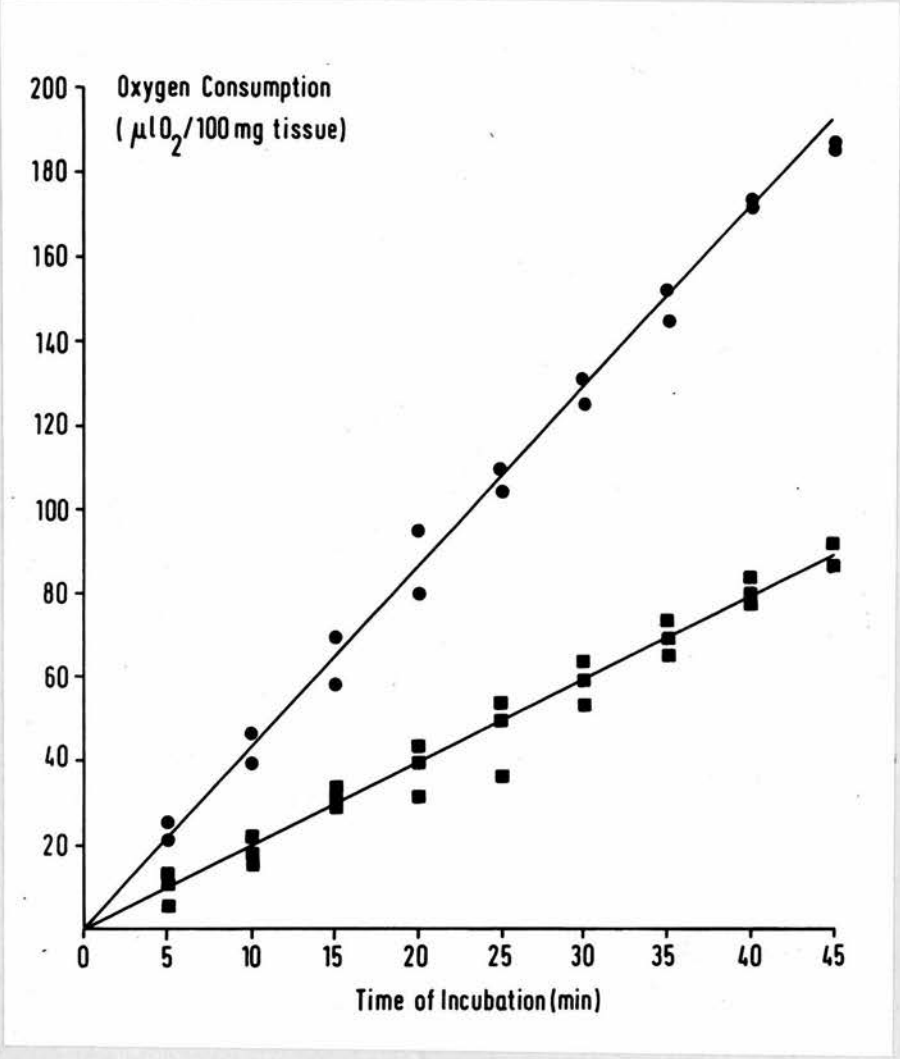


Fig. 3:1

Oxygen consumption of rabbit choroid plexuses incubated at 37°C for 45 mins in Krebs'-Ringer phosphate solution (Cremer, 1957) under an atmosphere of oxygen. Incubations were carried out both in the presence (●—●) and absence (■—■) of 2,4-dinitrophenol (DNP).

error of 1%. The efficiency of counting was determined using the 'channels ratio' method (Baillie, 1960). In the first channel the lower discriminator was set at 50 and the upper at 95 whilst in the second, which also had a lower discriminator setting of 50, the upper was at 1000. Both channels had an 8% gain. A graph of the ratio of the counts per unit time registered in channel one to the counts per unit time in channel two against the efficiency of counting, was used to determine the efficiency of the counting in the samples and enabled the number of disintegrations per minute of the samples to be calculated. The graph relating the efficiency of the counting to the 'channels ratio' had previously been constructed for the toluene scintillant and that particular scintillation counter. Counts due to 'background' were estimated by including in every series of samples two vials which only contained 4 ml methanol/ethanol and 10 ml of the toluene scintillant.

RESULTS

Manometric

The oxygen consumption of the choroid plexuses of the rabbit was linear over the 45 mins of incubation (Fig. 3:1) and in that period was 1.97 µl 0_2 per min/100 mg tissue. Addition of 2,4-dinitrophenol to the medium, to give a final concentration of

10^{-4}M increased the respiration of the choroid plexuses to 4.31 ul O_2 per min/100 mg tissue, (Fig. 3:1).

Accumulation of ^{131}I-iodide by the isolated choroid plexus

The accumulation of ^{131}I-iodide by the lateral ventricular choroid plexuses of the rabbit is shown in Table 3:2. The results have been expressed as the ratio (T/M) of the counts per unit time per mg (wet weight) of tissue to the counts per unit time per µl medium and give a measure of the ability of the tissue to concentrate the iodide ion over and above the concentration found in the medium.

The accumulation was the same when 0.5 or 1.0 µCi of carrier-free ^{131}I-iodide was present in the medium (Table 3:2) but it was reduced by 43% in the presence of 10^{-4}M 2,4-dinitrophol (Table 3:2).

A consideration of the factors influencing estimates of T/M ratios

In the above experiment the T/M ratio was expressed as the ratio of the counts per unit time per mg (wet weight) of tissue to the counts per unit time per µl of medium, as this was the method used by Welch (1962a) and a direct comparison with his results was wanted. Ideally, the T/M ratio should be a measure of the ability of the cells of a tissue to concentrate a substance and should be derived from the ratio of the concentration of the free substance within the cells to the concentration outside the cell.

The quantity of the substance (x) within the cells of the tissue is the total quantity of x in that tissue minus the quantity

TABLE 3:2

Accumulation of carrier-free ^{131}I-iodide by the lateral ventricular choroid plexuses of the rabbit. The T/M value is the ratio of the radioactivity per mg tissue to that per µl incubation medium after 1 hour incubation at 30°C.

Activity of carrier-free ^{131}I-iodide (µCi) per ml incubation medium	T/M (Tissue/Medium ratio)
1.0	31.3 ± 1.9 (4)+
0.5	28.6 ± 1.6 (4)
1.0 + 10^{-4}M dinitrophenol	17.8 ± 0.4 (4)

+ Mean value ± Standard Deviation (No. of estimates).

in the extracellular space. If free diffusion of x in the extracellular space is assumed then its concentration in that phase will be the same as that in the medium. The volume of this extracellular space can be measured by use of a substance which can move freely in this phase but does not enter the cells, for example inulin. When inulin is used the extracellular space is known as the inulin space.

The quantity of x in the intracellular space can thus be derived from:-

The quantity of x/mg (wet weight) tissue (t) - (the quantity of x/µl medium (m) X the inulin space/mg (wet weight) tissue (i))

$$= t - mi \qquad (1)$$

If it is assumed that x exists free within the cell then the volume containing it will be the volume of intracellular water, which is:-

The weight of water/mg (wet weight) tissue - the inulin space/mg (wet weight) tissue (i).

The weight of water/mg (wet weight) tissue was derived from the value for the dry weight of the rabbit choroid plexus, quoted by Welch (1962a), of 15.07 $\pm$ 1.07% of the wet weight. This value was not determined independently as the expenditure of rabbits for this purpose was felt to be unjustified.

The volume of water per mg (wet weight) tissue is thus

0.85 µl and the volume of the intracellular water is:-

$$0.85 - i \qquad (2)$$

From equations (1) and (2) the concentration of x in the intracellular water is thus

$$\frac{t - mi}{0.85 - i}$$

As stated above, if free diffusion of x in the extracellular space is assumed then the concentration of x in that phase can be taken as equal to the quantity of x/µl medium (m) and the T/M value thus becomes:-

$$\frac{t - mi}{m(0.85 - i)}$$

This corrected T/M value was used in all experiments involving ^{14}C-5-HIAA.

Estimation of inulin space

The choroid plexuses of the lateral and fourth ventricles of the rabbit were incubated at 30°C in the physiological salt solution containing inulin for periods of time ranging from 10 to 80 mins.

At equilibrium the concentration of inulin in the extracellular fluid was taken to be the same as that in the medium. The volume of the extracellular space per unit wet weight of tissue is thus

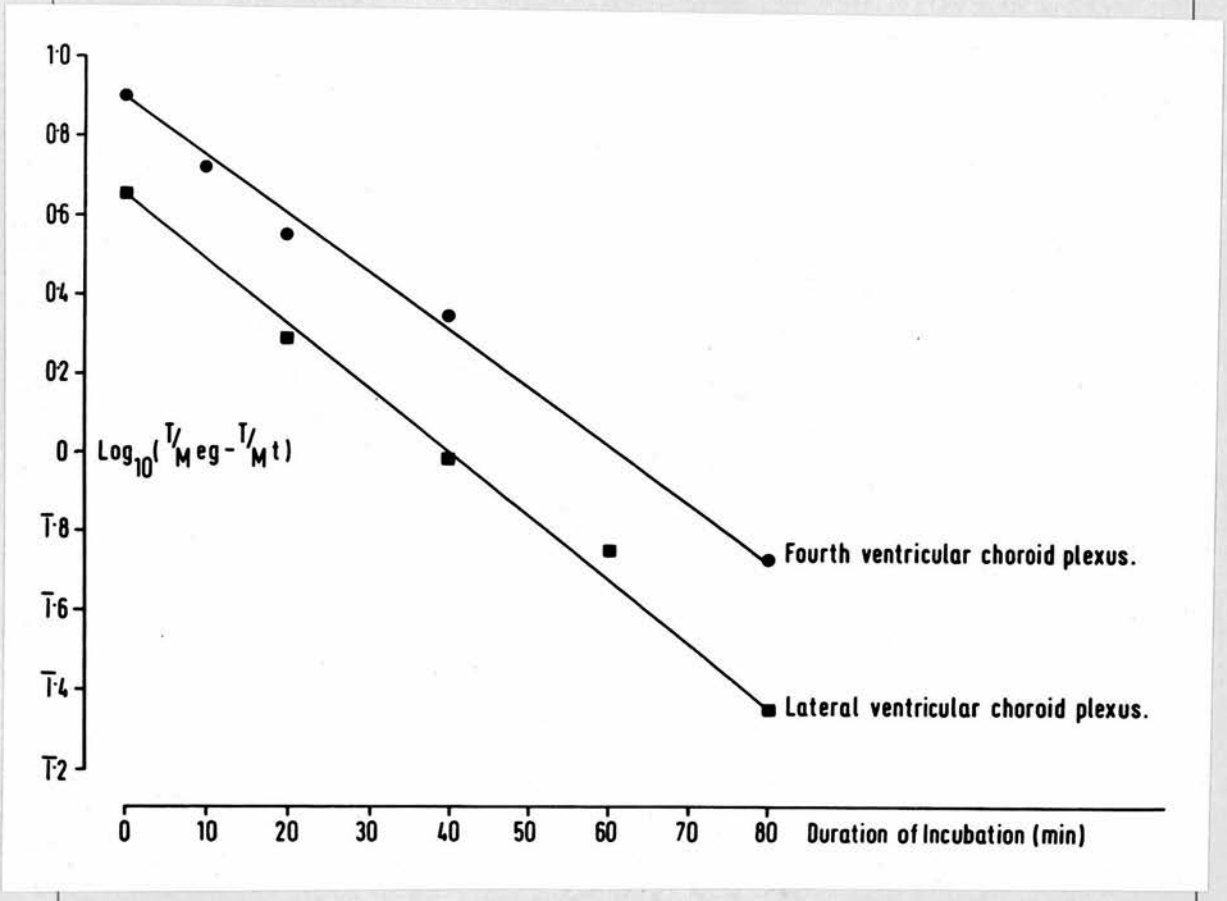


Fig. 3:2

The figure illustrates logarithmic plots of values of (T/M)eq minus the values of (T/M)t for the accumulation of 5-hydroxyindol-3-ylacetic acid (5-HIAA) by the lateral and fourth ventricular choroid plexuses of the rabbit when incubated at 30°C in a medium containing 200 ng/ml ^{14}C-5-HIAA (see Fig. 3:3).

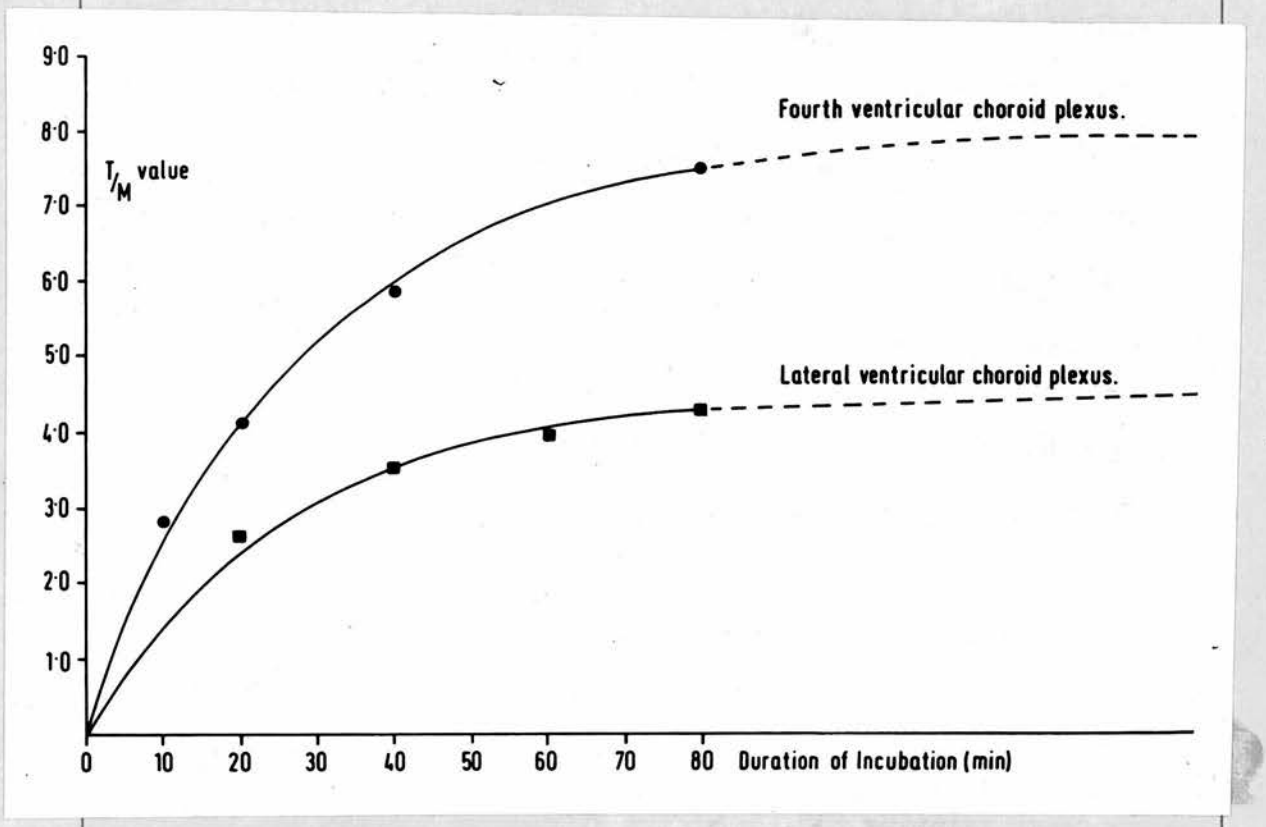


Fig. 3:3

The accumulation of 5-hydroxyindol-3-ylacetic acid (5-HIAA) by the lateral and fourth ventricular choroid plexuses of the rabbit when incubated at 30°C in a medium containing 200 ng/ml ^{14}C-5-HIAA.

given as:-

$$\frac{\text{The equilibrium amount of inulin/mg (wet weight) tissue}}{\text{The amount of inulin/}\mu\text{l medium}}$$

The values obtained for the inulin space of the lateral and fourth ventricular choroid plexuses are given in Table 3:3. The inulin space of the fourth ventricular choroid plexus was found to be greater than that of the lateral ventricular choroid plexuses and the inulin reached equilibrium concentrations in this choroid plexus more rapidly.

The accumulation of ^{14}C-5-hydroxyindol-3-ylacetic acid by the isolated choroid plexuses of the rabbit

The lateral and fourth ventricular choroid plexuses of the rabbit were found to accumulate ^{14}C-5-HIAA when incubated at 30°C in a medium containing 200 ng ^{14}C-5-HIAA/ml (Table 3:4). The exponential nature of this process is shown by the linearity of the graph relating the log of the difference between the T/M value at equilibrium, (T/M) eq, and the T/M value at time t, (T/M) t, to the time of incubation (Fig. 3:2). The values of (T/M) eq were obtained by extrapolation of a graph relating the mean T/M values for the lateral and fourth ventricular choroid plexuses to the time of incubation (Fig. 3:3). The T/M value at equilibrium, (T/M) eq, for the choroid plexus from the fourth ventricle was found to be 1.8 times greater than that for the

TABLE 3:3

Inulin space of the lateral and fourth ventricular choroid plexuses.

Time of incubation (min)	Inulin space per mg (wet weight) tissue of the choroid plexuses from:-	
	lateral ventricles	fourth ventricle
10	0.14 ± 0.01 (4)+	0.26 ; 0.26
20	0.16 ± 0.01 (4)	0.26 ; 0.26
40	0.16 ± 0.01 (4)	0.26 ; 0.26
60	0.17 ± 0.01 (4)	0.25 ; 0.26
80	0.17 ± 0.01 (4)	0.26 ; 0.26

+ Mean value ± Standard Deviation (No. of estimates).

TABLE 3:4

Accumulation of ^{14}C-5-hydroxyindol-3-ylacetic acid by the lateral and fourth ventricular choroid plexuses of the rabbit. The tissue was incubated at 30°C in a medium containing 200 ng/ml ^{14}C-5-HIAA.

Time of incubation (min)	Tissue/Medium ratio (T/M) for choroid plexuses from:-	
	lateral ventricles	fourth ventricles
10	-	2.80
20	2.64 ; 2.57	4.16
40	3.24 ; 3.84	5.85
60	3.95 ± 0.85 (12)+	-
80	3.36 ; 5.19	7.48

+ Mean T/M ratio ± Standard Deviation (No. of estimates).

tissue from the lateral ventricles.

The T/M value for the accumulation of ^{14}C-5-HIAA by the lateral ventricular choroid plexuses was dependent on the concentration of ^{14}C-5-HIAA in the incubation medium, (Table 3:5). An increase in the concentration of 5-HIAA in the medium from 50 ng - 5 µg/ml reduced the proportion of the 5-HIAA accumulated by 50%. The T/M value for the accumulation of ^{14}C-5-HIAA by the lateral ventricular choroid plexuses was also dependent on the temperature at which the incubation was carried out (Table 3:6). Incubation at 6°C reduced the accumulation of 5-HIAA by 75 - 80% of that at 30°C for three different medium concentrations of 5-HIAA.

The effect of 2,4-dinitrophenol on the accumulation of ^{14}C-5-hydroxyindol-3-ylacetic acid by the lateral ventricular choroid plexuses of the rabbit

The presence of 2,4-dinitrophenol (DNP) in a concentration of 10^{-4}M in the incubation medium inhibited the accumulation of ^{14}C-5-HIAA by the lateral ventricular choroid plexuses of the rabbit (Table 3:7). The degree of inhibition was similar over a 40 fold range of concentrations of ^{14}C-5-HIAA in the incubating medium and was on average 41%.

Inhibition of the accumulation of ^{14}C-5-hydroxyindol-3-ylacetic acid by 3-methoxy-4-hydroxyphenylacetic acid

The effect of 3-methoxy-4-hydroxyphenylacetic acid

TABLE 3:5

Effect of varying ^{14}C-5-hydroxyindol-3-ylacetic acid concentrations in the incubation medium on the T/M values for the accumulation of this acid by the lateral ventricular choroid plexuses of the rabbit. Tissue incubated for 60 mins at 30°C.

^{14}C-5-HIAA concentration in incubation medium (ng/ml)	T/M value
5000	2.07 ; 2.22
2000	2.17 ; 2.53
200	3.95 ± 0.85 (12)+
100	4.33 ; 4.46
50	4.54 ; 4.16

+ Mean value ± Standard Deviation (No. of estimates).

TABLE 3:6

Comparison of the accumulation of ^{14}C-5-hydroxyindol-3-ylacetic acid by the choroid plexuses from the lateral ventricles of a rabbit when incubated for 60 mins at 30°C and at 6°C.

Concentration of ^{14}C-5-HIAA in incubating fluid (ng/ml)	T/M value incubation at 30°C	T/M value incubation at 6°C	Average decrease of accumulation (% of 30°C T/M value)
200	3.95 ± 0.85 (12)[+]	0.79 ; 0.85	79
100	4.33 ; 4.46	1.06 ; 1.07	76
50	4.54 ; 4.16	1.32 ; 0.88	75

[+] Mean value ± Standard Deviation (No. of estimates).

TABLE 3:7

Inhibition by 2,4-dinitrophenol (DNP) of the accumulation of ^{14}C-5-hydroxyindol-3-ylacetic acid by the lateral ventricular choroid plexuses fo the rabbit. Incubation for 60 mins at 30°C.

Concentration of ^{14}C-5-HIAA in incubating medium (ng/ml)	T/M value		Average % inhibition
	No DNP	10^{-4}M DNP	
2000	2.17 ; 2.53	1.38 ; 1.57	37
200	3.95 ± 0.85 (12)+	2.38 ; 2.49	38
100	4.33 ; 4.46	2.07 ; 2.10	53
50	4.54 ; 4.16	2.70 ; 2.82	38

+ Mean value ± Standard Deviation (No. of estimates).

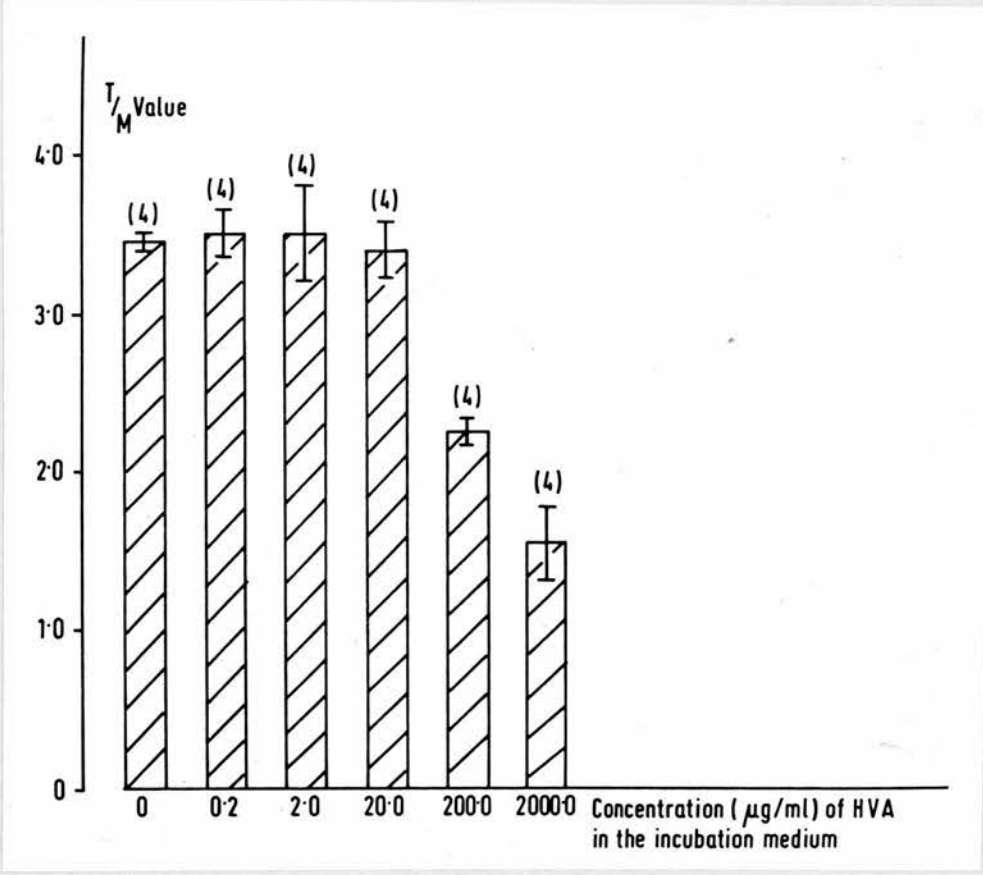


Fig. 3:4

Inhibition of the accumulation of 5-hydroxyindol-3-ylacetic acid (5-HIAA) by the lateral ventricular choroid plexuses of the rabbit when incubated at 30°C for 60 mins in a medium containing 200 ng/ml ^{14}C-5-HIAA and different concentrations of 3-methoxy-4-hydroxyphenylacetic acid (HVA). Each value represents the mean ± the standard deviation of four estimates.

(homovanillic acid, HVA), a normal constituent of c.s.f. (Anden, Roos and Werdinius, 1963) on the transport of 5-HIAA from the ventricular system of the dog *in vivo* was studied by Ashcroft *et al.*, (1968). These authors found that an increase in the concentration of HVA in the cerebral ventricles inhibited the removal of 5-HIAA. These two acids may, therefore, be removed from the c.s.f. *in vivo* by the same transport system for which they compete. The effect of increasing concentrations of HVA in the incubating medium on the accumulation of 5-HIAA by the lateral ventricular choroid plexuses of the rabbit was studied in order to determine whether it inhibited the transport of this latter acid *in* ~~vivo~~ *vitro*.

When the lateral ventricular choroid plexuses of the rabbit were incubated at 30°C for 60 mins in a medium containing HVA and 200 ng 5-HIAA/ml (approx. 10^{-6}M), there was no inhibition of the accumulation of this latter acid until the concentration of HVA in the medium was 200 µg/ml (approx. 10^{-3}M). At this concentration of HVA there was a 35% reduction in the T/M value (Fig. 3:4).

Inhibition of accumulation of 5-hydroxyindol-3-ylacetic acid with probenecid

Probenecid, which has been shown to inhibit the accumulation of 5-HIAA by kidney cortex slices (Despopoulous and Weisbach, 1957) also inhibits the removal of 5-HIAA from rat brain *in vivo*

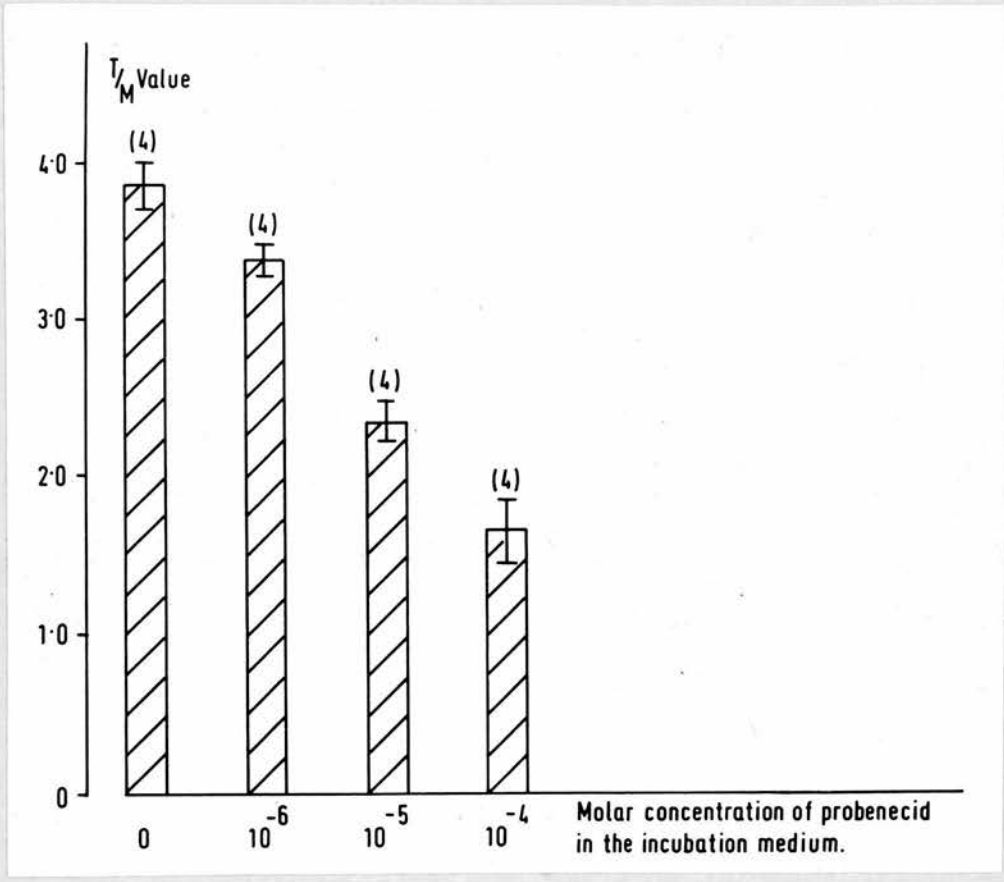


Fig. 3:5

Inhibition of accumulation of 5-hydroxyindol-3-ylacetic acid (5-HIAA) by the lateral ventricular choroid plexuses of the rabbit when incubated at 30°C for 60 mins in a medium containing 200 ng/ml ^{14}C-5-HIAA and different concentrations of probenecid. Each value represents the mean ± standard deviation of four estimates.

(Neff, Tozer and Brodie, 1967), and from a recirculatory perfusion of the ventricular system of the dog (Ashcroft et al., 1968). In the present study the effect of the presence of probenecid in the incubating medium on the accumulation of ^{14}C-5-HIAA by the lateral ventricular choroid plexuses of the rabbit was assessed in order to determine whether this substance inhibited the uptake of 5-HIAA by this tissue.

When incubated at 30°C for 60 mins in the presence of probenecid in a medium containing 200 ng/ml ^{14}C-5-HIAA (approx. 10^{-6}M), the accumulation of the latter substance by the lateral ventricular choroid plexuses of the rabbit was inhibited, (Fig. 3:5). A reduction in the T/M value of more than 50% was obtained when the concentration of probenecid in the medium was 10^{-4}M (28.5 µg/ml).

DISCUSSION

The concentration gradient which exists between the blood and the cerebrospinal fluid (c.s.f.) for a large number of substances may, in part, be due to the removal of these substances from the c.s.f. at a greater rate than their rate of entry from the blood (Pollay 1966; Cutler, Robinson and Lorenzo, 1968). That a removal mechanism does exist has been shown, using the technique of ventricular perfusion, for diodrast and phenolsulphonphthalein in

the goat (Pappenheimer et al., 1961), for p-aminohippuric acid (PAH) in the cat (Davson et al., 1962), for PAH, thiocyanate and iodide in the rabbit (Pollay and Davson, 1963), and for 5-HIAA and HVA in the dog (Ashcroft et al., 1968). The position of the choroid plexuses and the similarity of their epithelial cells to those of the proximal tubule of the kidney (Davson, 1967) has led a number of workers to implicate them in this transport system and to investigate, in vitro, their ability to accumulate various substances against a concentration gradient by a mechanism which is dependent on metabolic energy. In particular, the isolated choroid plexuses of the rabbit have been used and have been shown to be capable of the active accumulation of iodide, thiocyanate, sulphate, thiosulphate, 5-hydroxytryptamine, noradrenaline, decamethonium, hexamethonium, N'-methylnicotinamide, morphine and morphine derivatives (Welch, 1962a,b; Tochino and Shanker, 1965 a,b; Takemori and Stenwick, 1966; Hug, 1967; Robinson et al., 1968).

The method used in the present study to remove the choroid plexuses from the rabbit gave viable preparations which had an oxygen consumption of 1.97 µl O_2 per min/100 mg tissue (Fig. 3:1). These choroid plexuses were capable of accumulating carrier-free ^{131}I-iodide to the same extent as those used by Welch (1962a) to give a tissue concentration up to 30 times the concentration in the medium. Good agreement was also obtained with the T/M value of

27 observed by Robinson et al., (1968) after incubating the lateral choroid plexuses of the rabbit with ^{131}I-iodide for 1 hour.

When calculating the T/M value used in the present study (page 153), allowance was made for the ^{14}C-5-HIAA in the extracellular fluid of the choroid plexuses. The volume of the extracellular space was measured using inulin.

The inulin space of 0.17 for the choroid plexuses from the lateral ventricles and 0.26 for that from the fourth ventricle (Table 3:3) was lower than the value of approximately 0.3 measured by Welch (1962b) for the rabbit choroid plexuses and by Lorenzo and Cutler (1969) for those from the cat. The reason for this difference is unknown but it may lie in a lack of penetration of the inulin into the capillaries of the choroid plexuses used in the present study.

The lateral ventricular choroid plexuses of the rabbit, when incubated at 30°C for 60 mins in a medium containing 200 ng 5-HIAA per ml accumulated the 5-HIAA to give a final concentration in the intracellular water 3.95 $\pm$ 0.85 (S.D. : n = 12) times the concentration in the medium (Table 3:4). The mechanism for the accumulation of 5-HIAA was saturable for the T/M value was decreased as the concentration of 5-HIAA in the medium was increased. The T/M value was the same for concentrations of 5-HIAA in the medium of 50 ng and 100 ng per ml, with a 10% reduction in this value at a concentration of 200 ng per ml, (Table 3:5). The

system for the accumulation of 5-HIAA was thus saturated when the concentration of 5-HIAA in the incubation fluid was between 100 ng and 200 ng/ml.

The ability of the fourth ventricular choroid plexus of the rabbit to accumulate 5-HIAA was approximately 1.8 times greater than that of those from the lateral ventricles (Fig. 3:3). This is similar to the results obtained by Welch (1962a), who showed that the ratio of uptake of ^{131}I-iodide by the fourth ventricular choroid plexus to that by the lateral ventricles was 1.5. It is of interest that Tochino and Shanker (1965a,b) on studying the accumulation of the cationic substances 5-hydroxytryptamine, noradrenaline, decamethonium and hexamethonium by the choroid plexuses of the rabbit observed that those from the lateral ventricles accumulated these compounds to a greater extent than that from the fourth ventricle.

The dependence of the transport mechanism for 5-HIAA in the lateral ventricular choroid plexuses of the rabbit on ATP formation was shown by the reduction in T/M value produced by the introduction of 2,4-dinitrophenol (DNP) into the incubating medium to give a final concentration of 10^{-4}M (Table 3:7). DNP is a potent uncoupler of oxidative phosphorylation and that it was uncoupling oxidative phosphorylation in the concentration used in the present study was indicated by the ability of this concentration to increase the oxygen consumption of the choroid plexuses 2.2 times

(Fig. 3:1).

There thus appears to exist in the choroid plexuses of the rabbit a saturable mechanism capable of accumulating 5-HIAA against a concentration gradient and which is dependent on metabolic energy. Such a system could be said to be an "active transport" system.

Homovanillic acid (HVA), the major metabolite of the biogenic amine, dopamine, is a normal constituent of c.s.f., (Anden, Roos and Werdinius, 1963) and has been shown to be transported from the c.s.f. by a saturable transport mechanism (Ashcroft et al., 1968). These workers also found that an increase in the concentration of HVA in the c.s.f. inhibited the removal of 5-HIAA from the cerebral ventricles. The degree of inhibition observed was not however very large and the infusion of HVA into the ventricular system at a rate of 135 µg/min only increased the concentration of 5-HIAA in the c.s.f. from the cisterna magna by 25%. In the present study the effect of HVA on the accumulation of 5-HIAA by the lateral ventricular choroid plexuses was also small as the addition of HVA to the incubating medium containing 200 ng 5-HIAA/ml (approx. 10^{-6}M) did not inhibit the accumulation of the latter substance until the concentration of HVA was 200 µg/ml (approx. 10^{-3}M) (Fig. 3:4).

In the light of these results and also of the finding that HVA is rapidly removed from the cerebral ventricles in vivo (Ashcroft et al., 1968), it can be postulated that two transport

systems are involved in the removal of HVA and 5-HIAA from the c.s.f. If the system concerned with the removal of 5-HIAA also has a slight affinity for HVA then, when the concentration of HVA is sufficiently large in comparison with that of 5-HIAA, inhibition of the transport out of the c.s.f. would be observed. The existence of mutually independent transport systems for HVA and 5-HIAA is also suggested by the results, presented earlier in this thesis, for the estimations of the concentrations of these two acids in the lumbar c.s.f. of parkinsonian patients treated with L-DOPA. The administration of L-DOPA to these patients in some cases raised the concentration of HVA in the lumbar c.s.f. from approximately 10^{-7} to 10^{-5}M without causing any significant increase in the concentration of 5-HIAA (approx. 10^{-7}M). Further information about the interaction of HVA and 5-HIAA could have been obtained by studying the effect of 5-HIAA on the accumulation of HVA by the choroid plexuses. This experiment was not carried out as radioactively labelled HVA was not readily available from commercial sources.

Probenecid, which has been shown to inhibit the uptake of 5-HIAA by kidney cortex slices (Despopoulous and Weisbach, 1957), also inhibits the removal of 5-HIAA from rat brain *in vivo* (Neff, Tozer and Brodie, 1967), and from a recirculatory perfusion of the ventricular system of the dog (Ashcroft *et al.*, 1968). In the present study it has been shown that the accumulation of 5-HIAA by

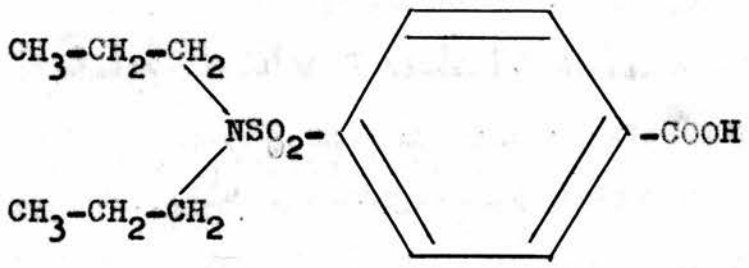


Fig. 3:6

Molecular formula of probenecid.

the lateral ventricular choroid plexuses of the rabbit is inhibited by the presence of probenecid in the incubation medium (Fig. 3:5). As probenecid is a monobasic phenolic acid (Fig. 3:6) it is possible that the inhibition produced by it is of a competitive nature.

The ability of the choroid plexuses from the lateral and fourth ventricles of the rabbit to accumulate 5-HIAA is limited as the equilibrium T/M values are small (4.5 and 8.0 respectively) when the tissue is incubated at 30°C in a medium containing 200 ng 5-HIAA per ml. The system might thus seem of little value in the removal of 5-HIAA from the c.s.f. *in vivo*. The *in vitro* system used here, however, gives no indication of the rate of transfer of 5-HIAA from the choroid plexuses into the blood. This rate might be expected to be high as the transfer of 5-HIAA from the cells of the choroid plexuses into the blood will be with a concentration gradient. Under these conditions there would be no net accumulation of 5-HIAA within the cells of the tissue. A better index of the ability of these structures to transfer 5-HIAA from the c.s.f. to the blood would therefore be the initial rate of entry of the acid into the choroid plexuses and not their T/M values at equilibrium. The initial rate of entry of 5-HIAA into the choroid plexuses when they are incubated in a medium containing 200 ng 5-HIAA per ml, can be calculated from the initial rise in the T/M values shown in Fig. 3:3. These are 0.17/min for the lateral

and 0.35/min for the fourth ventricular choroid plexuses. As the concentration of 5-HIAA in the extracellular space has been taken as being the same as in the medium (page/55) the initial rate of entry of this acid into the lateral ventricular choroid plexuses is 34 ng/g (wet weight) tissue/min and into that from the fourth ventricle is 70 ng/g (wet weight) tissue/min. In the rabbits used in the study each choroid plexus weighs approximately 10 mg, and if it is assumed that the choroid plexus from the third ventricle behaves in a similar manner to those from the lateral ventricles then the total initial rate of accumulation of 5-HIAA by all the choroid plexuses from the one animal would be 1.7 ng/min. If the in vivo situation is the same as the in vitro one then the initial rate of removal of 5-HIAA from the ventricular space of the rabbit by way of the choroid plexuses will be 1.7 ng/min which will equal the absolute rate of removal provided there is no accumulation of the acid in the cells of the tissue. In the dog the rate of removal of 5-HIAA by active transport is 16 ng/min (Ashcroft et al., 1968). It should be pointed out that there are difficulties associated with the above reasoning. The first lies in drawing a close parallel between the in vitro and the in vivo situation where no such parallel may exist. The second is in the assumption that the 5-HIAA does not accumulate within the cells of the choroid plexus. At physiological pH the 5-HIAA will be ionized and as such may not be capable of passing easily into the

blood. It would thus accumulate within the cells reducing the rate of entry of the 5-HIAA from the c.s.f. The work presented here, however, shows that 5-HIAA can enter the cells of the choroid plexuses at physiological pH and there is no reason to suppose that there does not exist a mechanism for its removal from these cells into the blood. Further insight into the transfer of 5-HIAA from the c.s.f. to the blood by way of the choroid plexuses could be obtained by perfusion of the vascular capillary system of this tissue.

The experimental work presented in this section demonstrates the point at issue, namely, that the choroid plexuses are a likely site for the active removal of 5-HIAA from the c,s.f. It however gives little information about the kinetics of this transport system.

APPENDIX

Biosynthesis of Tritiated

3-methoxy-4-hydroxyphenylethyleneglycol sulphate

Biosynthesis of tritiated 3-methoxy-4-hydroxyphenyl-ethyleneglycol conjugate.

There is evidence that the major catabolite of noradrenaline in brain is 3-methoxy-4-hydroxyphenyl-ethyleneglycol (MOPEG) which, in some species, is present as the conjugate, probably the sulphate ester (Schanberg, Breese, Schildkraut, Gordon and Kopin, 1968). This conjugate is found in human c.s.f. and in order to determine the concentration of MOPEG, liberation of the free glycol is necessary before its estimation, for example, by fluorimetry. Schanberg, Schildkraut, Breese and Kopin (1968) have demonstrated that the conjugate is susceptible to the action of sulphatase-containing preparations. For the development of a quantitative hydrolytic procedure, using such sulphatase-containing products as 'Helicase', it is essential to have a preparation of the conjugate in order to be able to monitor the degree of hydrolysis under varying conditions. A radioactively labelled preparation is preferable in such circumstances in order to allow differentiation from endogenous material should it be desired to measure the extent of hydrolysis in solutions containing the latter.

A biosynthetic preparation of the conjugate of the tritiated glycol was obtained by isolation from rat brain after the injection of ^{3}H-normetanephrine into the cerebral ventricles. This conjugate has been demonstrated by Schanberg, Schildkraut, Breese and Kopin (1968) to be the major catabolic product of normetanephrine which had been injected into the cisterna magna of rats. Peripheral administration is unsatisfactory because, like the parent amine the O-methylated derivative does not readily enter the central nervous system and peripheral metabolism is mainly directed to the acid catabolite 3-methoxy-4--hydroxymandelic acid. The O-methylated derivative labelled with tritium was used in preference to ^{14}C-labelled noradrenaline, although the latter label is the more desirable, in order to avoid the necessity of separating the labelled O-methylated glycol from the non--methylated labelled glycol derivative.

Radioactive normetanephrine Normetanephrine-7-^{3}H (3.9 Ci/m mole) was obtained from New England Nuclear Corporation as a solution in 0.1N acetic acid and was prepared for injection as follows.

To one ampoule containing 0.25ml 0.1N acetic acid was added 0.1ml 0.1N sodium hydroxide and 1.15ml of artificial c.s.f. (Table). The resultant solution had a pH of 7.4.

TABLE

Composition of artificial c.s.f.

	concentration (g/l)
NaCl	8.98
KCl	0.25
$CaCl_2$	0.14
$MgCl_2$	0.11
NaH_2PO_4	0.07
Urea	0.13
Glucose	0.16

Injection of normetanephrine The labelled normetanephrine was introduced as described by Noble, Wurtman and Axelrod (1967) into a lateral ventricle of six male Wistar rats.

The animals were lightly anaesthetised with ether, a midline sagittal incision made from the eyes to the ears and the skin parted using gentle pressure with the fingers. A hole, large enough to take a No27 gauge needle and deep enough to penetrate the skull, was made with a thin metal probe, 1.5-2.0 mm lateral to the crossing of the sagittal and coronal sutures. Using a 50ul Hamilton syringe fitted with a 27 gauge needle with a stop 3.5-4.0 mm from its tip, 20µl of the labelled normetanephrine solution (3.3 µCi) was injected into the lateral ventricle. The needle was allowed to remain in place for 5 seconds and was then withdrawn. The incision was closed with skin clips.

Isolation of MOPEG conjugate One hour after the injection the rats were killed by decapitation and the brains rapidly removed and weighed. Each brain was homogenised separately in an all glass, Potter type, homogeniser in ice cold 0.4N perchloric acid (4 ml/g tissue) and the protein precipitate removed by centrifugation at 18,000g for 5 min. The supernatant from the homogenate was adjusted to pH 4.0 (glass electrode) with 5N and 1N

potassium hydroxide and kept at -20°C overnight. The solutions were brought to about 4°C and the precipitate of potassium perchlorate was sedimented by centrifugation at 18,000g for 5 min at 4°C.

Unchanged normetanephrine was adsorbed from each supernatant by passing it through a 5 cm long, 5 mm diameter column of Dowex 50 resin, 200-400 mesh, Na^+ form. The effluent from the column was collected in a 30 ml glass stoppered test tube and adjusted to pH 1.0 (glass electrode) with 2N HCl. Phenolic acidic and unconjugated glycol metabolites were extracted from the aqueous solution into ethyl acetate by shaking three times in all for 5 min with 10 ml of ethyl acetate. The phases were separated each time by centrifugation at 3,000g for 5 min and the upper organic layer discarded. The MOPEG conjugate remains in the aqueous phase because, being a relatively strong acid, it remains fully ionised at pH 1.0.

The aqueous solution was taken to dryness under reduced pressure (about 0.5 mm Hg) at 40°C. The residue from each brain was taken up in 0.3 ml of methanol and transferred by replicate applications to the origin of a 2.5 cm wide strip of Whatman No 1 chromatography paper. Markers of MOPEG and the acid metabolite of normetanephrine vanillin mandelic acid (VMA), were applied to a separate strip. The chromatograms were developed by descending

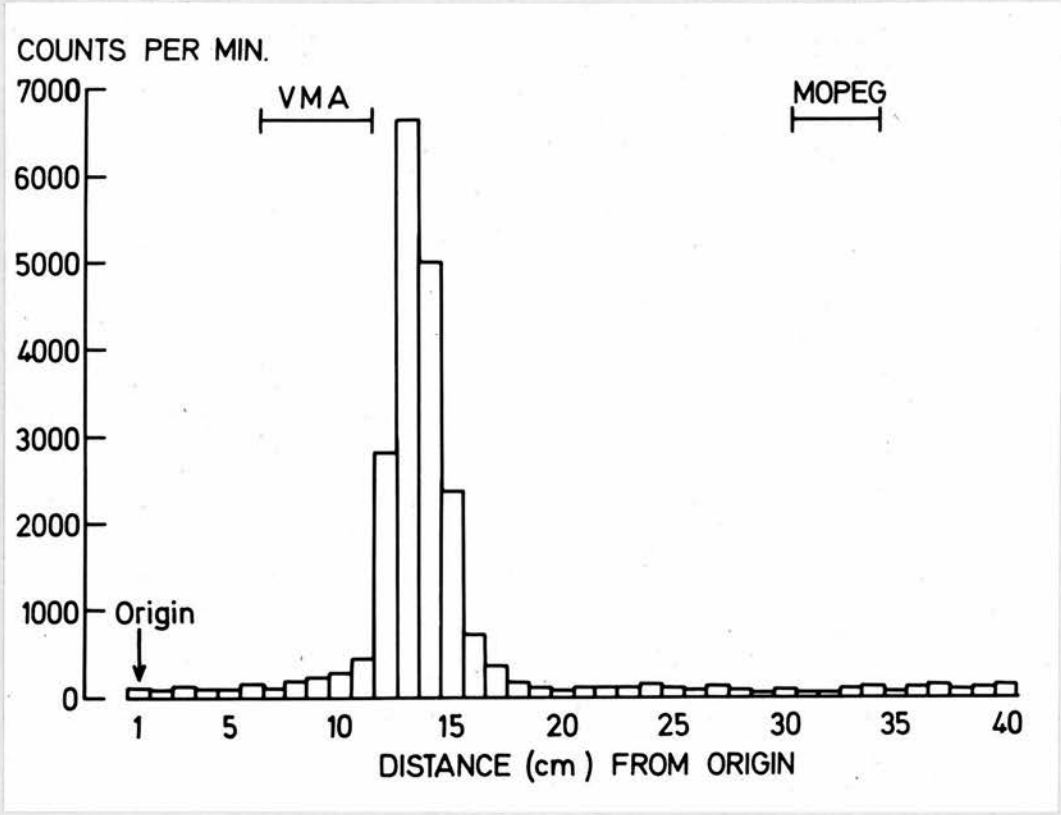


Fig. 1

Distribution of radioactivity on a chromatogram of a solution believed to contain the conjugate of tritiated 3-methoxy-4-hydroxy-phenylethyleneglycol extracted from rat brain after the administration of normetanephrine-7-^{3}H intraventricularly.

Descending chromatography on Whatman No 1 paper for 7 hr in chloroform methanol: ammonia (sp.gr. 0.88) (12:7:1, v/v).

chromatography for 7 hr in chloroform: methanol: ammonia (sp.gr. 0.88) (12:7:1 by volume) (Sugden, Yates and Eccleston, unpublished). The developed chromatograms were dried in a fume cupboard in a stream of air. To locate the radioactive material on the chromatogram, a strip 0.5 cm wide was cut from one edge and divided into 1 cm lengths which were placed in liquid scintillator vials. Methanol (1.0 ml) was added to each paper segment to elute any ^{3}H-MOPEG sulphate. After 1 hr, 10 ml of toluene scintillant (page 144) was added to each vial and the activity estimated with a Nuclear Chicago Mark II liquid scintillation counter.

A histogram showing the distribution of radioactivity along a typical chromatogram is shown in Fig. 1. The radioactivity on the chromatogram gave a single peak, the position of which did not correspond to that of MOPEG or of VMA on the marker strip, the position of which had been visualised by spraying the strip with diazotised p-nitroanaline.

The sections of paper containing the remaining radioactivity on the chromatograms were cut out and each stored in a 30 ml glass stoppered test tube at 4°C until the radioactive material was eluted from them in water.

Identification of radioactive material from the chromatogram If the radioactivity on the chromatogram was derived from MOPEG conjugate, then after treatment with the sulphatase-containing preparation, 'Helicase', it should become extractable into ethyl acetate and show the chromatographic mobility of MOPEG.

The radioactive material in the appropriate segment of the developed chromatogram was eluted by gently shaking the piece of paper for 20 min with 12 ml water in a 30 ml glass stoppered test tube. An aliquot (0.5 ml) of this eluate was taken for the estimation of tritium by liquid scintillation counting. The remainder of the eluate was divided into two 5.0 ml samples in 15 ml glass stoppered test tubes. To one sample was added 1.0 ml 1M acetate buffer pH 5.0, and to the other, 1.0 ml of the buffer containing 20 mg of the sulphatase preparation 'Helicase'. In order to prevent bacterial growth, one drop of chloroform was added to each tube and the samples were gassed with nitrogen prior to incubation at 37°C for 16 hr.

The incubated solutions were cooled to room temperature and the protein precipitated from each by the addition of 0.2 ml zinc sulphate (40% w/v $ZnSO_4$ $7H_2O$) followed by 0.15 ml 20% w/v sodium hydroxide. The precipitates were removed by centrifugation for 5 min at

3,000 g and the supernatants transferred to 30 ml glass stoppered test tubes, adjusted to pH 1.0 with 2N HCl and saturated with sodium chloride. The samples were shaken for 5 min with 10 ml of ethyl acetate and, after centrifugation at 3,000 g for 5 min, 7 ml of the upper organic layer was transferred to a 100 ml round bottomed flask. The extraction was repeated twice with further 10 ml volumes of ethyl acetate which were pooled in the round bottomed flask with that from the first extraction. A 1.0 ml portion from each combined ethyl acetate extract was taken for the estimation of radioactivity by liquid scintillation counting.

Of the radioactivity present in the sample treated with the sulphatase preparation 'Helicase', 87% was extractable into the ethyl acetate as compared with 2% for the sample incubated in the absence of 'Helicase'.

Chromatography of ethyl acetate extract The ethyl acetate extract from the sample which had been incubated with 'Helicase' was evaporated to dryness under a stream of nitrogen. The residue was dissolved in 0.3 ml methanol and transferred, by replicate applications, to the origin of a 2.5 cm wide strip of Whatman No 1 chromatography paper. Marker amounts of authentic MOPEG and VMA were applied to a second strip and both were developed, by

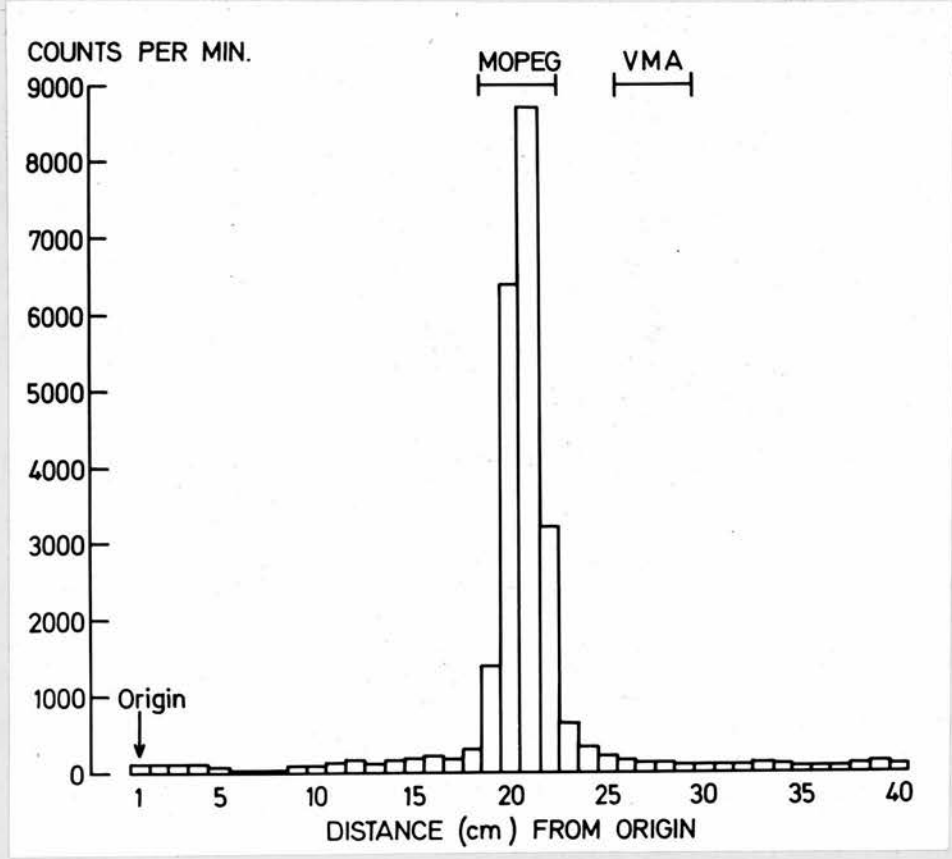


Fig. 2

Chromatographic distribution of ethyl acetate extractable radioactivity following incubation with the enzyme preparation 'Helicase', of a brain extract believed to contain the conjugate of tritiated 3-methoxy-4-hydroxyphenylethyleneglycol.

Descending chromatography on Whatman No 1 paper for 6 hr with isobutylmethylketone: 4% (v/v) formic acid (10:1 v/v).

descending chromatography, in the organic phase of the solvent, isobutylmethylketone: 4% (v/v) formic acid (10:1 v/v) (Guldberg, 1967) for 6 hr. The developed chromatograms were dried in a fume cupboard in a stream of air. The MOPEG and VMA in the marker chromatograms were visualised by spraying with diazotised p-nitroaniline (page 28). The chromatogram containing the radioactivity was cut into 1 cm wide strips along the direction of the solvent flow. Each paper segment was cut into smaller pieces which were placed in a liquid scintillation vial. Methanol (1.0 ml) was added to each vial to elute any radioactivity present. After 1 hr, 10 ml of toluene scintillant was introduced into each vial and the radioactivity estimated using a Nuclear Chicago Mark II liquid scintillation counter.

The radioactivity on the chromatogram was found to be located in a single discrete area corresponding in position to MOPEG on the marker chromatogram (Fig. 2).

It would appear from its solvent extraction characteristics and chromatographic behaviour before and after enzymatic hydrolysis that the radioactivity in the purified fraction, isolated from rat brain after injection of ^{3}H-normetanephrine, was indeed located only in material which had the characteristics of endogenous MOPEG conjugate. It was considered that the evidence was

sufficient to justify the use of the material, without further purification, to monitor the enzymatic hydrolysis of MOPEG conjugate.

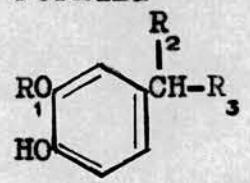
Formula			Name	Abreviation used in text
				
R_1	R_2	R_3		
H	H	CH_2NH_2	Dopamine	-
H	OH	CH_2NH_2	Noradrenaline	-
H	OH	CH_2NHCH_3	Adrenaline	-
CH_3	H	CH_2NH_2	Methoxytyramine	-
CH_3	OH	CH_2NH_2	Normetanephrine	-
CH_3	OH	CH_2NHCH_3	Metanephrine	-
H	H	COOH	3,4-dihydroxyphenylacetic acid	DOPAC
H	OH	COOH	3,4-dihydroxymandelic acid	DOMA
CH_3	H	COOH	3-methoxy-4-hydroxyphenylacetic acid (Homovanillic acid)	HVA
CH_3	OH	COOH	3-methoxy-4-hydroxymandelic acid (Vanillin mandelic acid)	VMA
H	H	CH_2OH	3,4-dihydroxyphenylethanol	DOPET
H	OH	CH_2OH	3,4-dihydroxyphenylethyleneglycol	DOPEG
CH_3	H	CH_2OH	3-methoxy-4-hydroxyphenylethanol	MOPET
CH_3	OH	CH_2OH	3-methoxy-4-hydroxyphenyl-ethyleneglycol	MOPEG

TABLE OF FORMULAE AND ABBREVIATIONS.

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