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Glycol metabolites of noradrenaline in brain tissue

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1. A gas chromatographic method using electron capture detection is described for the estimation of those catecholamines and their metabolites which possess one or two free alcoholic hydroxyl groups. It is based on acetylation of phenolic hydroxyl and primary and secondary amino groups in aqueous solution, extraction into a water immiscible solvent and esterification of alcoholic hydroxyl groups with heptafluorobutyric anhydride.

2. The method has been successfully applied to the detection and estimation of free 4-hydroxy-3-methoxyphenylethylene glycol (MOPEG) and free 3,4-dihydroxyphenylethylene glycol (DOPEG) in hypothalamic brain tissue.

3. Although it was possible to measure noradrenaline and normetanephrine by the same method in pure solution, crude tissue extracts could not be used, despite their suitability for estimating the glycol metabolites.

Mannarino, Kirshner & Nashold (1963) showed that when (±)-β-^{14}C-noradrenaline was injected into the lateral cerebral ventricle of the cat, it was metabolized in the brain to 4-hydroxy-3-methoxyphenylethyleneglycol (MOPEG) and 3,4-dihydroxyphenylethyleneglycol (DOPEG). Glowinski, Kopin & Axelrod (1965) injected tritiated noradrenaline into the cerebral ventricles of the rat and also found that the major part of the deaminated metabolites formed in the brain consisted of MOPEG. The presence of a sulphate conjugate of MOPEG in normal rat brain was reported by Schanberg, Schildkraut, Breese & Kopin (1968) but these authors were unable to detect the free glycol in this species. Recently, Schanberg, Breese, Schildkraut, Gordon & Kopin (1968) have described the determination of MOPEG in brain tissue and cerebrospinal fluid using the method described for urine by Wilk, Gitlow, Clarke & Paley (1967). In this method a trifluoroacetyl derivative of MOPEG is formed and measured by gas chromatography using the electron capture detector.

The formation of acetyl and perfluoroacyl derivatives as the basis of methods for the estimation of catecholamines and their metabolites has been described by Brooks & Horning (1964), Horning, Horning, Vanden Heuvel, Knox, Holmstedt & Brooks (1964) and Wilk, Gitlow, Franklin & Carr (1964). The main problem in applying these methods to biological samples lies in the difficulty of extracting small quantities of catecholamines and their metabolites from tissues and then achieving the anhydrous conditions required for the introduction of perfluoroacyl and acetyl groupings without excessive losses.

Chattaway (1931) showed that phenolic hydroxyl groups and amino groups could be acetylated very easily in dilute aqueous alkaline solution. This reaction was used

by Welsh (1955), Hagopian, Dorfman & Gut (1961) and Laverty & Sharman (1965) as a step in the isolation of catecholamines and their metabolites from aqueous solutions and tissue extracts. The acetylated derivatives are more stable than the parent compounds and thus can be brought more easily to the anhydrous state. Alcoholic hydroxyl groups are not acetylated in these conditions and are left free to form a perfluoroacyl derivative. This paper describes the use of these steps in an approach to the problem of estimating catecholamines and their metabolites in tissues and body fluids by gas chromatography.

Methods

The following chemicals and reagents were used: bis (4-hydroxy-3-methoxyphenylethyleneglycol) piperazine salt (Calbiochem); 3,4-dihydroxyphenylethyleneglycol (Calbiochem); (−)-3,4-dihydroxyphenylethanolamine ((−)-noradrenaline) base (Hoechst A.G.); (±)-4-hydroxy-3-methoxyphenylethanolamine ((±)-normetanephrine Calbiochem); 4-hydroxy-3-methoxyphenylethanol (prepared by reduction of 4-hydroxy-3-methoxyphenylacetic acid with lithium aluminium hydride); γ-benzene hexachloride (Shell); heptafluorobutyric anhydride. Prepared from heptafluorobutyric acid (Minnesota Mining and Manufacturing Co. Ltd.) by heating under reflux with phosphorus pentoxide (1 mol. heptafluorobutyric acid: 1·5 moles P_2O_5) for 3 hr. Distilled three times, B.P. 108° C (uncorrected); dichloromethane (May & Baker Ltd., distilled three times); tetrahydrofuran (Hopkin & Williams, distilled fresh in small quantities as required); acetic anhydride (British Drug Houses Ltd., distilled twice, stored at 4° C); methanol (James Burrough Ltd., purified by distillation from KOH and then redistilled); hexane (Hexane fraction, Hopkin & Williams, distilled twice). All other chemicals were of analytical reagent quality. Double distilled water was used throughout.

Extraction of tissues

The tissues were removed rapidly after killing the animals and homogenized in ice-cold 0·1 N hydrochloric acid (4 ml. acid for up to 0·5 g tissue). Concentrated perchloric acid (72% w/v), 0·2 ml., was added for each 4 ml. of homogenate and mixed thoroughly. Solid potassium chloride was then added in a slight excess of the amount required for saturation. The homogenate was then centrifuged with a tip radial acceleration of 147000 ms^{-2} (15000 *g*) for 5 min at 0° C. The clear supernatant was transferred to a glass stoppered test tube and mixed with 0·3 ml. acetic anhydride. A slight excess of sodium bicarbonate was added in small portions with mixing, allowing the effervescence to subside between additions. The solution was then extracted twice with 10 ml. portions of dichloromethane which were dried by filtering through anhydrous sodium sulphate. The extract was evaporated to a small volume, under a stream of dry nitrogen, in test tubes standing in holes in an electrically heated aluminium block at 60° C. The concentrated extract was then transferred to a 75 mm × 8 mm borosilicate glass test tube and evaporated to dryness at 60° C under a stream of dry nitrogen.

Formation of heptafluorobutyrate esters

The sample was dissolved in a small volume (20–200 μl.) of tetrahydrofuran. One quarter of this volume of heptafluorobutyric anhydride was then added and mixed.

The mixture was allowed to stand at room temperature for 5–10 min and then evaporated to dryness under a stream of dry nitrogen at 60° C. The gas flow and heating were continued for 15 min after the liquid had disappeared. The formation of the acetylheptafluorobutyryl derivatives is thought to proceed as shown in Fig. 1.

Gas chromatography

This was carried out on both Model 400 and Model 402 F & M Scientific Corporation gas chromatographs using in the former a U-shaped column 180 cm × 3 mm internal diameter packed with 3·8% SE-30 on silanized Diatoport S (60–80 mesh). This column had been conditioned at 300° C for 12 hr and then used at 230° C for several months for the analysis of steroid compounds. The Model 402 was fitted with a new 120 cm × 4 mm internal diameter column of 3·8% SE-30 on Diatoport S conditioned by heating at 300° C for 12 hr.

The carrier gas was argon containing 5% methane. A tritium foil electron capture detector was used with both instruments.

The derivatives were dissolved in hexane or in a hexane solution of γ-benzene hexachloride of known concentration for injection into the gas chromatograph. The extracts prepared from tissues were dissolved in volumes of 0·1–0·5 ml. of which 0·5–2 μl. were injected.

Results

Formation of heptafluorobutyryl derivatives of noradrenaline and some catecholamine metabolites

4-Hydroxy-3-methoxyphenylethyleneglycol (MOPEG) was separated from the piperazine in the commercially available salt by dissolving in water, adding hydrochloric acid to bring the pH to 1–2 and extracting three times with five volumes of diethyl ether. The ether extracts were dried with anhydrous sodium sulphate and evaporated under a stream of nitrogen, yielding an oily residue. The heptafluorobutyryl derivatives of both MOPEG and acetyl-MOPEG were prepared from known

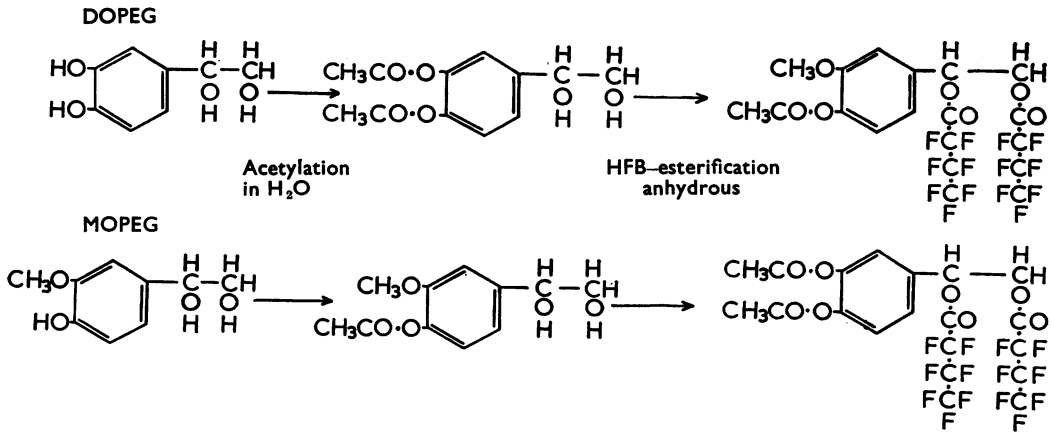

FIG. 1. Formation of acetyl-heptafluorobutyryl derivatives of DOPEG and MOPEG.

amounts of MOPEG. Figure 2 illustrates the separation of these two derivatives on the gas chromatogram and shows that the fully heptafluorobutyrated derivative did not give a response larger than the acetyl-heptafluorobutyryl derivative when these were prepared by the methods described here.

Acetyl-heptafluorobutyryl derivatives were prepared from (−)-noradrenaline, (±)-4-hydroxy-3-methoxyphenylethanolamine (normetanephrine) MOPEG, DOPEG and 4-hydroxy-3-methoxyphenylethanol. The derivatives of noradrenaline, MOPEG and DOPEG were crystallized at −17° C from hexane. The uncorrected melting points of these derivatives were: acetylheptafluorobutyryl MOPEG, white crystals, 75·5°–76° C; acetyl heptafluorobutyryl DOPEG, white crystals, 71°–72° C; acetyl-heptafluorobutyryl noradrenaline, white needles, 93° C.

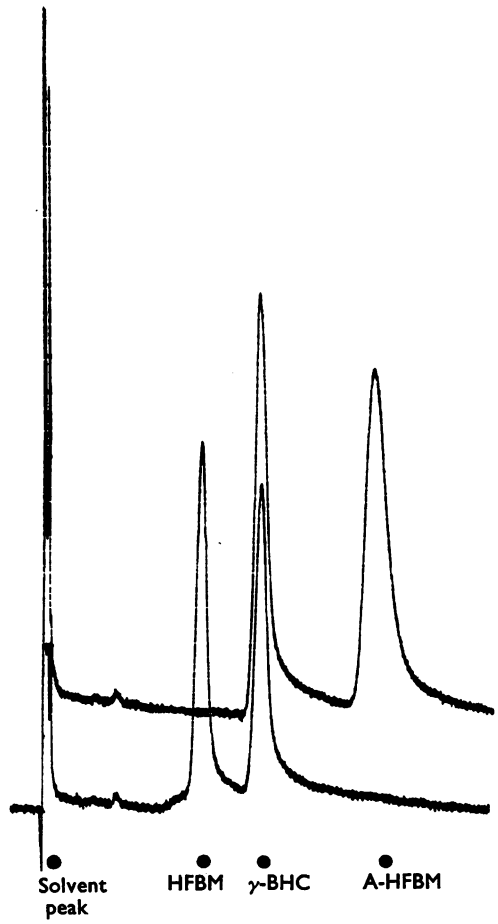

FIG. 2. Gas chromatogram tracings of the fully heptafluorobutyrated derivative of 4-hydroxy-3-methoxyphenylethyleneglycol (MOPEG) and the acetyl-heptafluorobutyryl derivative of MOPEG. The responses were obtained with mixtures containing γ-benzene hexachloride and derivatives of MOPEG in the proportions given in parentheses. γ-BHC, γ-benzenehexachloride (4 parts); HFBM, heptafluorobutyryl MOPEG (=7 parts MOPEG); A-HFBM, acetylhepta-fluorobutyryl MOPEG (=8 parts MOPEG). Chromatogram conditions: column temperature 125° C; flash heater temperature 160° C; E.C. detector temperature 180° C; gas flow 100 ml./min at 1·4 kg/cm^2 pressure; E.C. detector pulse interval 50 μsec; column 180 cm × 3 mm 3·8% SE-30.

The retention times of these derivatives relative to γ-benzene hexachloride in differing conditions for chromatography are given in Table 1. Each can be separated from the others on a 3·8% SE-30 column. However, the relative retention time of the noradrenaline derivative is too long at column temperatures below 150° C to allow the measurement of small amounts of noradrenaline and at higher temperatures the response from the derivative of MOPEG tends to overlap with that of the benzene hexachloride, the reference compound. The hexane solutions of the acetyl-heptafluorobutyryl derivatives of MOPEG, DOPEG, normetanephrine and noradrenaline were stable for several months at −17° C if care was taken not to allow water to condense inside the vessels holding the solutions when portions were taken out. The crystalline derivatives were not very stable unless kept under hexane at −17° C.

Quantitative estimation of MOPEG and DOPEG

The principle of the method has been described for the estimation of steroid compounds by Vandenheuvel, Hinderks, Nixon & Layng (1965), Wisniewski & Umbreit (1965) and by Heap, Holzbauer & Newport (1966); it is based on the ratio of the sizes of the responses of two substances, *a* and *b* which have different retention times. If the ratio of the sizes of the two responses is proportional to their relative concentrations in a mixture of the two substances and the total amount present in the sample of one of them is known, then the total amount of the other substance present in the sample can be calculated from the equation

$$A = \frac{RA}{RB} \times B \times C$$

where A is the total amount of substance *a* present in the sample, RA, the size of response to *a* in the sample, RB, the size of response to *b* in the sample, B, the amount of substance *b* present, and C the ratio b/a of responses to equal amounts of *a* and *b*.

The last term in the equation is obtained by calculation from the responses observed when samples each containing known amounts of the derivatives both of authentic A and of authentic B are tested on the gas chromatogram.

In the present experiments benzene hexachloride was selected as the reference

TABLE 1. *Relative retention times of heptafluorobutyryl esters of noradrenaline and some related compounds*

	Retention times relative to that of γ-benzene hexachloride				
Column temperature (°C):	127	127	134	140	152
Carrier gas flow (ml./min):	100	100	100	60	100
Compound					
1. Heptafluorobutyryl esters of					
4-hydroxy-3-methoxyphenylethanol	0·53				
4-hydroxy-3-methoxyphenylethyleneglycol (MOPEG)	0·63				
2. Acetyl-heptafluorobutyryl esters of					
4-hydroxy-3-methoxyphenylethanol	1·05				
4-hydroxy-3-methoxyphenylethyleneglycol (MOPEG)	1·33	1·46	1·31	1·40	1·15
3,4-dihydroxyphenylethyleneglycol (DOPEG)		2·81	2·31	2·50	1·92
2-(4-hydroxy-3-methoxyphenyl)ethan-2-olamine (normetanephrine)		3·88	3·33	3·60	2·78
2-(3,4-dihydroxyphenyl)ethan-2-olamine (noradrenaline)					4·62

compound to be added to the extracts. Figure 3 shows that the ratios of the heights of the response peaks obtained with the acetyl-heptafluorobutyryl derivatives of MOPEG and DOPEG and γ-benzene hexachloride are suitable for such estimations because the peak height ratios are proportional to the concentration ratios. When peak height ratios were replaced by ratios of areas of the peaks (calculated by triangulation) the variance of the relation between concentration ratio and response ratio was higher because two measurements were required for each area estimation.

Recoveries. Authentic MOPEG was added to tissue homogenates in quantities ranging from 10 to 50 ng to show that any MOPEG present in samples would be detected on the gas chromatogram. In the earlier experiments it was difficult to estimate MOPEG accurately without having the crystalline derivative to obtain accurate standard measurements. In two experiments with the procedure finally adopted in which crystalline standards were used the recovery of MOPEG was 64% and 69% and of DOPEG 67% and 78%.

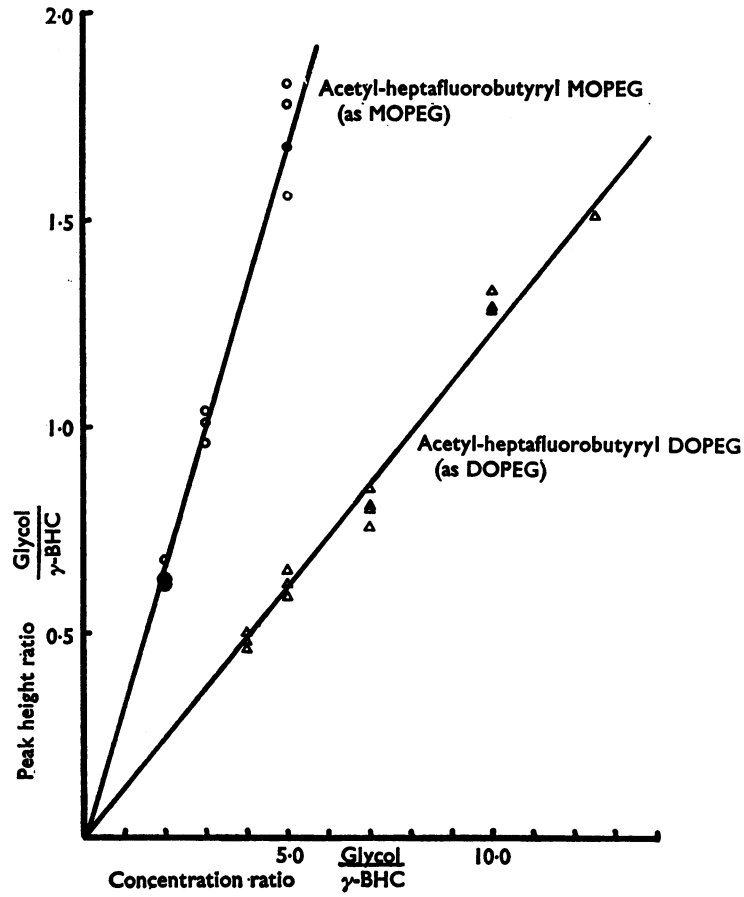

FIG. 3. Ratio of the peak heights of the responses to γ-benzene hexachloride and the acetyl-heptafluorobutyryl derivatives of MOPEG and DOPEG plotted against the ratio of their concentrations in mixtures. (The concentrations of the derivatives of the glycols are expressed as the concentration of the parent glycol.)

Presence of MOPEG and DOPEG in the hypothalamus

Hypothalamic tissues of the mouse, rabbit and cat were extracted and the extracts treated to form acetyl-heptafluorobutyryl derivatives. The gas chromatographic record obtained with an extract of mouse hypothalamus is shown in Fig. 4. This shows that the extract of mouse hypothalamic tissue gives response peaks similar to those of authentic derivatives of MOPEG and DOPEG. The retention times relative to that of the response due to γ-benzene hexachloride are identical with those obtained with the acetyl-heptafluorobutyryl derivatives of MOPEG and DOPEG. The responses correspond, in this experiment, to a concentration in the tissue of MOPEG 0·04 μg/g and DOPEG 0·05 μg/g (uncorrected for recovery). Similar peaks were seen on the recordings obtained with the extracts of rabbit (Sharman, 1969) and cat hypothalamus.

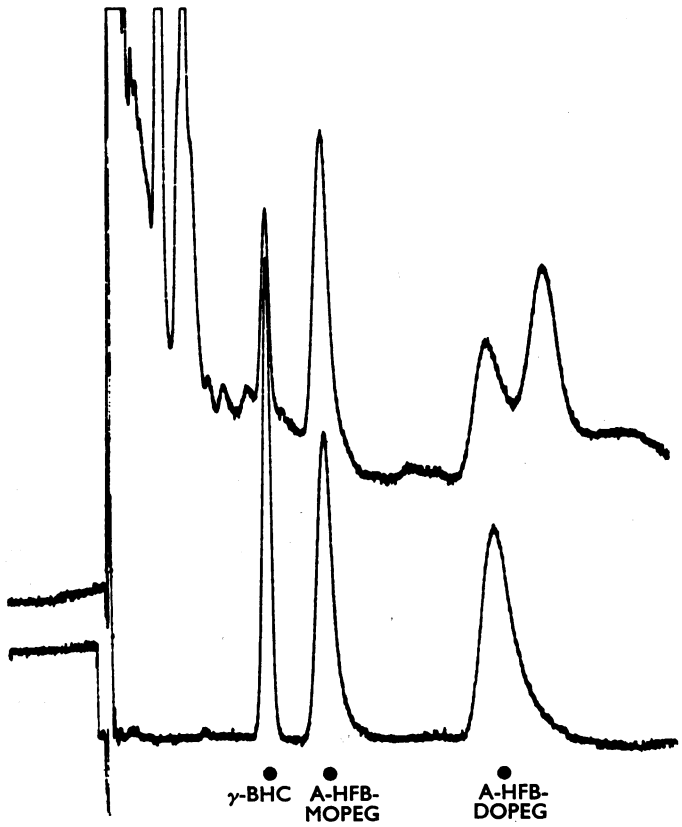

FIG. 4. Gas chromatographic record of an extract of mouse hypothalamus. Upper tracing: Mouse hypothalamic extract (from 0·103 g tissue) to which 1·25 ng γ-benzene hexachloride had been added. Lower tracing: Responses to derivatives of authentic MOPEG and DOPEG. Ratio of concentrations BHC:MOPEG:DOPEG=200:250:500 ng/ml. Column temperature 140° C; flash heater temperature 180° C; detector temperature 170° C; carrier gas flow 60 ml./min (argon: 5% methane; no purge gas). Pulse interval 150 μsec. Retention times relative to that of γ-benzene hexachloride observed with the extract used for the above record (Mean ± S.E.M. from three observations). Authentic derivatives of (1) MOPEG, 1·357 ± 0·004; (2) DOPEG, 2·443 ± 0·002. Peaks observed in extract (1) MOPEG, 1·367 ± 0·006; (2) DOPEG, 2·440 ± 0·08.

Further evidence for the identity of the substances giving rise to the peaks on the chromatograph record

Hydrolysis of the acetyl derivative of MOPEG

As shown in Fig. 2, the responses of the heptafluorobutyryl derivatives of MOPEG and acetyl-MOPEG are easily distinguished. The acetyl group on the phenolic hydroxyl of MOPEG, introduced during the extraction procedure, can be removed by dissolving in an alkaline aqueous solution. After evaporation to dryness the MOPEG can then be treated to yield the completely heptafluorobutyrylated derivative. A dichloromethane extract containing the acetylated derivatives was prepared from rabbit hypothalamus and from rabbit brain cortex to which MOPEG had been added. Each of these was divided into two equal portions and the four samples evaporated to dryness. To one of each pair of samples was added 0·1 ml. of concentrated ammonia solution and after 5 min these were again evaporated to dryness. All four samples were then treated with heptafluorobutyric anhydride in tetrahydrofuran as described and the final extracts examined by gas chromatography. A peak corresponding with that given by acetyl-heptafluorobutyryl MOPEG was observed with the two extracts that had not been treated with ammonia. The other two extracts showed a response at the retention time corresponding to that of heptafluorobutyryl MOPEG but the size of the response was difficult to estimate because it coincided with the sharp rising phase of another peak on the chromatogram and was observed as a shoulder on this peak.

Paper chromatographic separation of the acetyl derivatives before gas chromatography

A dichloromethane extract containing the acetylated derivatives was prepared from 1·1 g of mouse hypothalamus. One-tenth of this extract was taken, evaporated to dryness, treated to form the acetyl-heptafluorobutyryl derivatives and examined on the gas chromatograph. Peaks corresponding with those obtained with acetyl-heptafluorobutyryl derivatives of both MOPEG and DOPEG were obtained similar to those in Fig. 4. The remainder of the dichloromethane extract was evaporated to a volume of approximately 0·3 ml. This was applied to 1·5 cm wide lanes of Whatman No. 50 filter paper for chromatography which had been washed with 2 N sodium hydroxide and distilled water as described by Sharman (1963). A descending chromatogram was run using a modification of the Bush solvent system C (Bush, 1952). The solvent system was toluene : methanol : water : ethyl acetate in the proportions 10 : 5 : 5 : 2 and the chromatogram was developed for 2·5 hr at 27° C after equilibrating overnight. After development, the chromatogram was dried and cut up into 1 cm long portions each of which was eluted by intermittent shaking with 1 ml. of methanol which was left in contact with the paper for 1 hr. The pieces of paper were removed, the eluates evaporated to dryness and then treated to form the acetyl-heptafluorobutyryl derivatives. They were then examined by gas chromatography. The result is illustrated in Fig. 5. This shows that those responses on the gas chromatogram at relative retention times similar to those of the acetyl-heptafluorobutyryl derivatives of MOPEG and DOPEG, are only obtained from regions of the chromatograms where acetyl-MOPEG and acetyl-DOPEG are found.

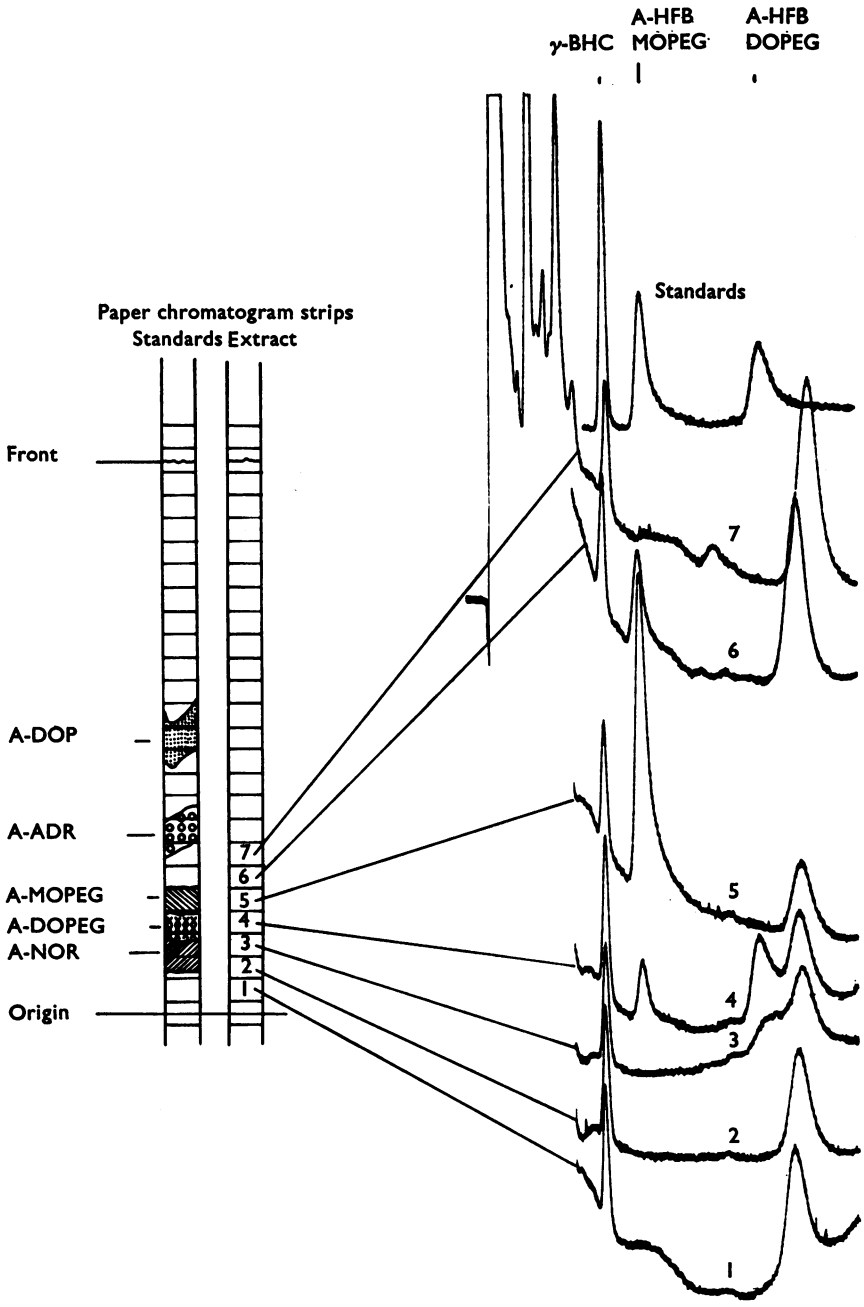

FIG. 5. Gas chromatographic records obtained with eluates from a paper chromatogram of an extract of mouse hypothalamus. The catecholamines and their metabolites in the tissue were acetylated and separated as indicated on the paper chromatogram by the reference compounds shown in the diagram. The chromatogram was cut into small pieces as indicated by the transverse lines and each piece eluted with methanol. The eluates were evaporated and treated to form heptafluorobutyryl derivatives. No response peaks at retention times relative to that of γ-benzene hexachloride corresponding with those of acetyl-heptafluorobutyryl MOPEG and acetyl-heptafluorobutyryl DOPEG were observed with eluates from regions of the chromatogram other than those shown. The conditions for gas chromatography were similar to those given in Fig. 3. A-DOP, acetyl dopamine ; A-ADR, acetyl adrenaline ; A-MOPEG, acetyl MOPEG ; A-DOPEG, acetyl DOPEG ; A-NOR, acetyl noradrenaline ; γ-BHC, γ-benzene hexachloride ; A-HFB-MOPEG, acetyl-heptafluorobutyryl MOPEG ; A-HFB-DOPEG, acetyl- heptafluorobutyryl DOPEG.

Noradrenaline and normetanephrine

The estimation of these two substances after formation of their acetyl-heptafluorobutyryl esters using gas chromatography is theoretically possible because good responses are obtained when aqueous solutions of either are subjected to the extraction and estimation procedures described for MOPEG and DOPEG.

In practice, the application of the method to measurement of noradrenaline and normetanephrine in tissues has met with technical difficulties. When gas chromatograms of extracts of mouse and rabbit hypothalamus were developed at 140° C and below, a response with a relative retention time similar to that given by the acetyl-heptafluorobutyryl derivative of normetanephrine was observed. However, when the derivative prepared from authentic normetanephrine was added to such extracts, the retention time of the response given by the added normetanephrine derivative could just be distinguished from that of the response seen with the extract alone. Preliminary experiments on eluates from the appropriate regions of the chromatogram used to obtain the results described in Fig. 5 yielded responses, the retention times of which approximated to those of the acetyl-heptafluorobutyryl derivatives of noradrenaline and normetanephrine; but it has not yet been possible to confirm or disprove the identity of these retention times with those of the authentic derivatives. These results indicate that simple extraction procedures are not suitable for the estimation of the two amines by gas chromatography of their acetyl-heptafluorobutyryl derivatives. Moreover the noradrenaline and normetanephrine derivatives are relatively insoluble in hexane which adds to the difficulties in applying the method as given here.

Sensitivity of the method

Figure 4 shows the responses given by amounts of authentic derivatives corresponding to 50×10^{-12} g MOPEG and 100×10^{-12} g DOPEG. The responses given by the derivatives of normetanephrine and noradrenaline were, respectively, at least 20 times and 100 times smaller than that given by the derivative of MOPEG.

Stability of the final extracts

The final hexane extract prepared from a sample of rabbit hypothalamus was tested for its content of acetyl-heptafluorobutyryl MOPEG three times over a period of 4 days. The extract was kept at $-17°$ C between the tests. On the first day the content of MOPEG was estimated to be 9·6 ng, on the second 9·7 and 9·8 ng and on the fourth day 8·2 ng.

TABLE 2. *Concentration of MOPEG and DOPEG in the hypothalamus*

Species	MOPEG (μg/g)	DOPEG (μg/g)
Rabbit	0·045	Present but not estimated because of lack of standard
	0·047	
	0·159	0·030
	0·049	0·120
	0·048	0·126
Mouse	0·049	0·048
	0·077	0·054
	0·040	0·047
	0·048	0·029
Cat	0·018	0·022
	0·017	0·018

Estimated in duplicate portions of same homogenate

The observations on mouse tissue were made on pooled samples from six animals. The observations on rabbit and cat tissue were made on samples from individual animals. The duplicate estimations were made on pooled tissues using samples of homogenate equivalent to the tissue from one animal.

Concentration of MOPEG and DOPEG in the hypothalamus

Table 2 shows the concentration (uncorrected for recovery) of MOPEG and DOPEG estimated to be present in the hypothalamus of several species.

Discussion

A technique for the estimation of certain catecholamines which contain a secondary alcoholic hydroxyl group and of some of their metabolites has been developed using a double esterification procedure. The first, an acetylation in aqueous solution, converts the compound into derivatives which are soluble in a water-immiscible organic solvent and the second, the introduction of one or more heptafluorobutyryl groups, forms derivatives which can be detected by electron capture. The method has proved to be extremely sensitive for the glycol metabolites of noradrenaline. Although it is theoretically possible to use a similar procedure for noradrenaline and adrenaline and their methoxylated amine metabolites, technical problems have arisen which no doubt can be overcome by further purification of extracts of tissues.

The method for the estimation of the glycol metabolites measures the free glycols present in the tissues and yields values for the concentrations of DOPEG and MOPEG which are not very different in the three species examined. Schanberg, Breese, Schildkraut, Gordon & Kopin (1968) have also used a perchloric acid protein precipitation with results indicating that there is little if any hydrolysis of conjugated MOPEG during this procedure. Preliminary experiments have shown that the simple extraction procedure used here is difficult to apply after treatment of brain extracts with a sulphatase because of the presence of contaminants in the enzyme preparation. Whether the present method is more sensitive than the method of Wilk *et al.* (1967) will only be decided by a direct comparison but the sensitivity given by these authors (91,000 mm^2 for 10^{-9} moles) for the response to trifluoroacetylated MOPEG can be compared with approximately 5,000,000 mm^2 for 10^{-9} moles, a typical response with the present method.

In addition to confirming the presence of free MOPEG in the central nervous system, the present results show that free DOPEG is also present, a result predicted by experiments on the metabolism of radioactive noradrenaline in the brain.

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