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A method for the estimation of the catecholamines and their metabolites in brain tissue

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Summary

1. A method is described whereby the catecholamines, their precursors and metabolites may be estimated in the same brain sample. The method, outlined below, was rigorously tested in pure solution before being applied to the estimation of the catechol and methoxyamines in extracts of rat brain. (i) The brain tissue was homogenized in perchloric acid and the perchloric acid removed as its insoluble potassium salt. (ii) The acid metabolites of the amines were extracted from the acidified extract into ethyl acetate. (iii) After extraction with ethyl acetate, the aqueous extract was treated with acetic anhydride and sodium hydrogen carbonate and the acetylated derivatives of the amines extracted into dichloromethane. (iv) The aqueous solution remaining after extraction with dichloromethane contains the acetylated amino-acids. Acetyl-dopa could be extracted into dichloromethane only after acidification of this aqueous solution. The acetyl-dopa in this dichloromethane extract could be estimated fluorimetrically. (v) The dichloromethane extract containing the acetylated amines was chromatographed on paper in the organic phase of a mixture of toluene : ethyl acetate : methanol : water, 10 : 1 : 5 : 5. (vi) The acetylated amines were eluted and estimated fluorimetrically. A new method was developed for the estimation of 3-methoxytyramine and its acetylated derivative.

2. The reproducibility of the method was demonstrated by the consistent estimates obtained for the concentrations of noradrenaline, dopamine and 3-methoxytyramine in whole rat brain. Adrenaline and metanephrine were not detected. The concentration of normetanephrine in rat brain was at the limit of detection of the method.

3. Although the estimation for the levels of endogenous amines in whole rat brain were consistent, the recoveries of exogenous amines taken through the procedure were low and very variable. Control experiments demonstrated that the exogenous amines were lost at the perchloric acid extraction stage of the procedure.

Introduction

Many drugs have been shown to alter the concentrations of the catecholamines in tissues. However, investigations into the mode of action of such drugs have only become possible since the development of fluorimetric methods whereby not only the amines, but also their precursors and metabolites, can be determined in tissue

extracts. Because of difficulties in separating these compounds one from another, the number of amines and their metabolites which can be estimated in one sample has been limited. Deficiencies in separation have to some extent been made good by development of analytical methods which are sufficiently specific to allow one compound to be estimated in the presence of another and sufficiently sensitive to allow division of the sample into as many aliquots as there are compounds to be measured. Estimation of an amine or an amine metabolite in the presence of other related substances is not, however, always satisfactory if, as occurs in brain extracts, the concentration of the substance is low relative to the concentrations of other compounds present in the solution for assay. A method capable of separating and assaying the individual catecholamines, their precursors and metabolites in the one brain sample, thus avoiding the dangers attendant upon estimating one compound in the presence of others, is described in this paper. The least amount of amine or metabolite detectable in a sample is at the same time decreased because division of the sample into several portions is no longer necessary.

Methods

Materials

Unless otherwise stated, all reagents were of analytical grade. Distilled, de-ionized water and all-glass apparatus (cleaned in chromic acid and rinsed thoroughly) were used throughout.

1,2-Diaminoethane (ethylenediamine) (B.D.H. Lab. Reagent), distilled three times, was stored in a dark-glass bottle and used for not more than 2 weeks. Isobutanol (iso-butyl alcohol F.T. for the fluorimetric determination of vitamin B_1 B.D.H. Lab. Reagent) gave low blanks if the contents of one bottle were used within 2 weeks. Methanol (Analytical Reagent Grade) and toluene (Analytical Reagent Grade) were distilled once before use. Ethyl acetate (Analytical Reagent Grade) from a newly opened bottle (500 ml.) was used in each experiment. Hydrochloric acid (M.A.R. B.D.H.).

(−)-Adrenaline bitartrate (Burroughs Wellcome & Co.), (−)-noradrenaline bitartrate (Bayer Ltd.), dopamine hydrochloride (Koch-Light Lab.), dopa (DL-3,4-dihydroxyphenylalanine, Koch-Light Lab.), (±)-metanephrine hydrochloride (Calbiochem. B grade), (±)-normetanephrine hydrochloride (Calbiochem. B grade), 3-methoxytyramine (3-methoxy-4-hydroxyphenylethylamine, Calbiochem. B grade), tyramine hydrochloride (B.D.H. Lab. Chem.), 5-hydroxytryptamine (serotonin creatinine sulphate, Koch-Light Lab.), (±)-octopamine hydrochloride (K. and K. Lab. Inc.).

Extraction of brain tissue

The extraction technique was based on that described by Bertler, Carlsson & Rosengren (1958) and was developed using rat brain tissue.

Three white Wistar rats, weighing 100–150 g, were killed by decapitation. The brains (minus cerebelli) were wrapped in aluminium foil and frozen in solid carbon dioxide. They could be stored at −20° C overnight without any alteration in the concentrations of the catecholamines. The pooled brains were homogenized, while still frozen, with 0·4 N perchloric acid, 4 ml./g brain tissue, at 4° C in an all-glass

homogenizer in the presence of disodium edetate, 2 mg/g, and ascorbic acid, 500 μg/g. The homogenate was divided into three equal portions, each equivalent to 1·1 g rat brain, which were processed in parallel. Two of these portions served as duplicate samples for the estimation of the endogenous amines. To the third portion was added 200–800 mμg of each of adrenaline, noradrenaline, dopamine, metanephrine, normetanephrine and 3-methoxytyramine in order to determine the recovery of these substances through the analytical procedure. Each sample was centrifuged at 4° C for 7 min at 4,300 *g*, the supernatant removed and the protein precipitate re-extracted, at 4° C, with 0·4 N perchloric acid, 3 ml./g brain tissue. The two perchloric acid extracts from each sample were combined. Perchlorate was precipitated from the extract as the potassium salt, by the dropwise addition, with continuous shaking, of 5 N and 1 N-potassium hydroxide until the pH was between 3 and 4, using bromophenol blue (0·02 ml. of 0·02%, w/v) as internal indicator. The sample was stored at −20° C for at least 30 min and sometimes overnight, to allow maximum precipitation of potassium perchlorate and aggregation of any unprecipitated proteinaceous matter. The sample was thawed, centrifuged at 4° C for 7 min at 4,300 *g*, the supernatant removed and the precipitate washed with 0·5 ml. water at 4° C, centrifuged as before and the wash fluid added to the supernatant.

Removal of carboxylic acid metabolites

The supernatant was acidified to a pH between 1 and 2 by adding 0·1 to 0·2 ml. 2 N hydrochloric acid, and after saturation with sodium chloride it was shaken vigorously for 4 min with 1·5 vol. ethyl acetate. The extraction was repeated once. The concentrations of the acid metabolites were not determined in the present experiments. Methods for the estimation of homovanillic acid and 3,4-dihydroxyphenylacetic acid in such ethyl acetate extracts from brain samples are described by Guldberg & Yates (1969).

Acetylation procedure (Welsh, 1952)

After extraction of the acid metabolites into ethyl acetate, traces of this solvent remaining in the sample were removed by evaporation at room temperature under reduced pressure. The pH was adjusted to 4 with 5 N and 1 N potassium carbonate and the sample briefly centrifuged to sediment excess salt, which could make difficult the removal of dichloromethane in the subsequent extractions. The sample could be stored overnight, at −20° C, at this stage. The supernatant was taken off and the precipitate washed with 0·5 ml. water at 4° C. To the combined supernatant and wash fluid (about 6–8 ml.), containing the amino-acids and the amines, was added acetic anhydride (0·05 ml./ml. solution) followed immediately by sodium bicarbonate (0·1 g/ml. solution). The solution was stirred vigorously for 10 min before repeating the additions of acetic anhydride and sodium bicarbonate and stirring for a further 20 min. The acetylated amines were extracted by shaking the solution 3 times for 4 min each time, with 2 vol. dichloromethane. The dichloromethane extracts were combined and shaken briefly with 0·3 vol. 0·1 N hydrochloric acid to remove any unacetylated amines and then 4 times with 0·3 vol. water to remove the acid, and dried over anhydrous sodium sulphate. The extract was usually left overnight at this stage, at −20° C. The dichloromethane extract was decanted into a 100 ml. round-bottomed flask, the sodium sulphate residue washed with a further 5–10 ml. dichloromethane which was added to the main extract and

the combined extracts evaporated at room temperature to about 1 ml. under a jet of nitrogen. The sides of the flask were then carefully washed down with 2 ml. dichloromethane and the evaporation continued to dryness. For the subsequent chromatography the almost invisible residue was taken up in 0·3 ml. methanol for transfer to the paper, the flask being rinsed with a further 0·2 ml. methanol which was also applied to the paper.

The aqueous phase remaining from the acetylation procedure after extraction of the acetylated amines into dichloromethane would contain acetyl-dopa and any other acetylated amino acids such as acetyl-tyrosine.

Paper chromatography of acetylated amines

A sheet of Whatman No. 20 paper was cut into eight strips, 46 cm long and 2·5 cm wide (Fig. 1). The methanolic tissue extract was applied to a strip across the line marked "origin" (Fig. 1) so that the applied solution formed a band of not more than 1 cm width. A stream of nitrogen was directed on to the line of application to aid evaporation of the solvent. Four tissue extracts, applied to four separate strips, could be chromatographed simultaneously. Of the remaining four strips,

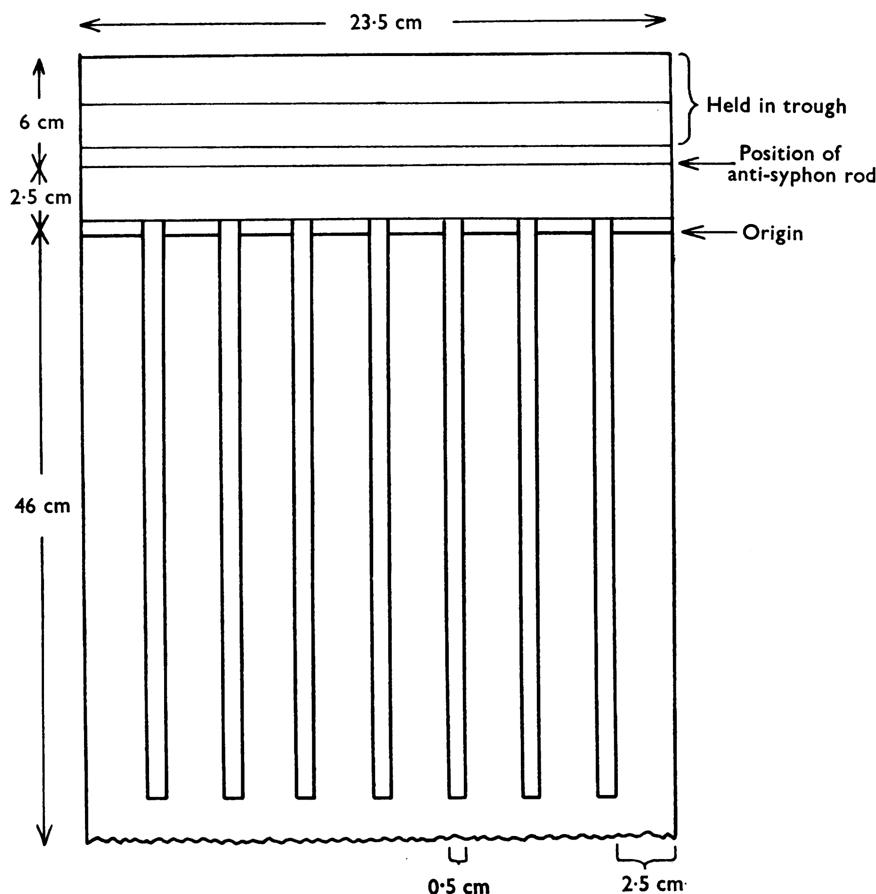

FIG. 1. Diagram of Whatman No. 20 paper sheet used for chromatography of the acetylated amines in toluene : ethyl acetate : methanol : water = 10 : 1 : 5 : 5.

three were used as markers and one, to which nothing was applied, was used for the preparation of paper blanks required for the fluorimetric assay of each amine. The marker strips were interspersed with the sample strips. An amount of extract equivalent to 1·25 g rat brain applied to the origin of a chromatogram was found not to affect the distances of flow of marker amounts of the acetylated amines, so these markers were chromatographed routinely in the absence of tissue extracts. The acetyl derivatives of adrenaline, noradrenaline, dopamine, metanephrine, normetanephrine and 3-methoxytyramine equivalent to 20–30 μg unacetylated amine were applied to the origin of each marker strip. The chromatogram was equilibrated for at least 6 hr in the presence of the organic and aqueous phases of a mixture of toluene : ethyl acetate : methanol : water (10 : 1 : 5 : 5 by vol., Bush, 1952). The chromatogram was developed in a descending direction with the organic phase for 10·5–11 hr at 19°–22° C. In order to obtain maximum separation of the amines, the duration and temperature of development were adjusted so that acetyl-3-methoxytyramine, the acetylated amine with the greatest R_f, reached close to the foot of the paper strips, the solvent dripping off the ends of the chromatograms. An all-glass tank was used; tanks with polythene accessories and plugs proved unsatisfactory because polythene absorbed the solvent vapours, causing leaks. Screw clamps and starch glycerine paste were used to ensure a tight seal between the tank and its lid. The tanks were lined with chromatography paper which was soaked in and dipping into the organic phase at the foot of the tank. A sheet of chromatography paper soaked in the aqueous phase was suspended from a trough, which was placed at the top of the tank and which contained the aqueous phase. The tanks were used once a week for 3 weeks after setting up and kept well sealed during this time. Organic and aqueous phases were replenished as required. Repeated use of the prepared tanks in this way appeared not to affect the separations adversely. The tank was enclosed in a large polystyrene box in order to protect the contents from draughts and ensure an even temperature within the tank. The slightest temperature gradient across the width of the tank resulted in differences in the rates of flow of the developing solvent down the chromatogram strips, with consequent differences in the distance travelled by a given acetylated amine on the individual strips. After development, the chromatograms were dried in an atmosphere of nitrogen and the amines on the marker strips visualized by spraying with a freshly prepared solution of diazotized *p*-nitroaniline followed by 5% (w/v) sodium hydroxide in 50% (v/v) methanol. The acetylated amines were eluted from the appropriate parts of the sample strips and blank strip in 15 ml. test tubes by gentle shaking for 30 min at room temperature in the following solutions: acetyl-adrenaline, acetyl-noradrenaline and acetyl-dopamine in 3·00 ml. water, acetyl-metanephrine and acetyl-normetanephrine in 2·00 ml. 0·4 N hydrochloric acid and acetyl-3-methoxytyramine in 2·00 ml. 0·1 N hydrochloric acid. The acetylated catecholamines were estimated within 24 hr and the acetylated methoxyamines within 4 days of their elution, the eluates being stored meanwhile at 4° C.

Preparation of standard solutions of the acetyl derivatives of the amines

20 μg of the substance in 5 ml. 0·005 N hydrochloric acid was acetylated and extracted as already described. The residue from the evaporated dichloromethane extract was dissolved in 10 ml. water to give a solution containing the equivalent

of 2 μg of the parent substance/ml. This solution was stored at 4° C and used as a standard solution in the course of the estimation of the parent substances in "unknown" solutions of the acetyl derivatives. Standards were prepared at monthly intervals and a new preparation was checked against the previous preparation before being used as a standard for assays. Standards prepared at monthly intervals over a period of 5 months gave the same readings when assayed together, demonstrating the reproducibility of the acetylation technique.

Solutions of the acetylated amines for use as markers were prepared in the same way, except that 15 mg of the substance was acetylated and the residue taken up in 1·5 ml. 50% (v/v) methanol to give a solution containing the equivalent of 10 mg/ml. unacetylated amine.

Blanks for fluorimetric estimations

Blanks prepared from a strip of paper to which nothing was applied and which was chromatographed in parallel with the sample strips were considered satisfactory for the following reasons: (i) The fluorescence from a "faded" blank (Brunjes, Wybenga & Johns, 1964) prepared from a brain chromatogram eluate known to contain acetyl-normetanephrine was the same as the fluorescence from the corresponding paper blank. (ii) Under normal conditions, adrenaline and metanephrine are not detectable in rat brain. As expected, the fluorescence readings obtained for these amines in brain chromatogram eluates were the same as those from blank paper chromatogram eluates. (iii) The same fluoresence was derived from those parts of a brain chromatogram and a blank paper chromatogram which did not correspond to any amine.

Estimation of acetylated derivatives of adrenaline, noradrenaline and dopamine

The method depended on the O-deacetylation of the acetyl derivatives and coupling with ethylenediamine to form a fluorophor (Weil-Malherbe & Bone, 1952).

To a 2·00 ml. sample containing the acetylated amine was added 0·50 ml. of a freshly prepared and cooled mixture of ethylenediamine and 4 M ammonium chloride, 1:1·3 by vol. (Kägi, Burger & Giger, 1957). The mixture was heated in a glass-stoppered test-tube at 50° C for 20 min with shaking, in a covered water-bath and, after cooling, it was saturated with sodium chloride and the fluorophor extracted into 2·00 ml. isobutanol (Weil-Malherbe, 1959) by shaking for 4 min. The relative fluorescence in the isobutanol extract was measured on the Aminco-Bowman spectrophotofluorimeter (SPF) at 430 mμ (activation)/500 mμ (fluorescence) for acetyl-adrenaline and acetyl-noradrenaline and at 430/520 mμ for acetyl-dopamine. The relative fluorescences of reagent blanks and aqueous standard solutions of the acetylated amines were determined concurrently with each batch of samples. Internal standards proved unnecessary, since eluates from brain chromatograms were found to have no effect on the fluorescence intensity derived from acetylated amine standards added in amounts comparable with those present in eluates from brain extracts.

Estimation of acetyl-3-methoxytyramine

As expected, acetylated 3-methoxytyramine, unlike acetylated dopamine, failed to yield a fluorophor on treatment with the ethylenediamine reagent, probably

because of the absence of a catechol grouping following O-acetyl hydrolysis in the alkaline medium. It was found, however, that the addition of periodate to a solution containing acetyl-3-methoxytyramine which had been heated in acid conditions, led to the formation of a fluorophor on subsequent reaction with ethylenediamine. Presumably, the periodate oxidatively demethylates (Adler & Magnusson, 1959) the N-acetyl-3-methoxytyramine produced by acid hydrolysis of the O-N-diacetyl-3-methoxytyramine to yield the catechol derivative, N-acetyl-dopamine, with which the ethylenediamine then interacts. The fluorophor thus produced had fluorescence characteristics identical to those obtained from acetyl-dopamine.

A 1·00 ml. portion of a solution of acetyl-3-methoxytyramine in 0·1 N hydrochloric acid (standard solution or chromatogram eluate) was heated for 20 min in a boiling water-bath. After cooling to room temperature, 0·50 ml. 0·1% (w/v) sodium periodate was added and the reaction allowed to proceed for 5 min under subdued lighting conditions. Ethylenediamine-ammonium chloride mixture, 0·50 ml., was added and the fluorophor developed and extracted into isobutanol as described for the acetylated catecholamines. Although the maximum fluorescence occurred at 430/520 mμ, the relative fluorescence was measured at 440/540 mμ because the blank contributed proportionately less to the fluorescence at the latter wavelengths than it did at the former. Reagent blanks and aqueous acetyl-3-methoxytyramine standards were run in parallel with each batch of samples. Internal standards proved unnecessary because eluates from brain chromatograms had no effect on the fluorescence from acetyl-3-methoxytyramine.

Eluates of blank paper chromatograms in 0·1 N hydrochloric acid gave fluorescence readings 3 to 5 times those from the reagent blanks, thus reducing the sensitivity of the method. A reduction of the paper blank to that obtained from ethylenediamine-treated eluates of the acetyl-catecholamine portions of the chromatogram was achieved by concentrating the acetyl-3-methoxytyramine by ascending chromatography in methanol into a much smaller area of the paper segment before elution. The " acetyl-3-methoxytyramine " section of the sample or blank chromatogram was sandwiched between two microscope slides with the end proximal to the origin of the chromatogram projecting 0·7 cm from the top of the slides (Fig. 2). The bottom of the slides was inserted into a glass trough containing methanol, so that the lower end of the paper strip was in line with the surface of the solvent. The solvent flowing up the paper evaporated from the exposed part of the strip but eventually reached the upper edge, when the flow was allowed to continue for a further 6 min. The strip was removed, allowed to dry and the top 0·7 cm eluted in 2·00 ml. 0·1 N hydrochloric acid. No acetyl-3-methoxytyramine was detected in the remainder of the strip in a control experiment in which the acetyl-3-methoxytyramine section of a chromatogram of a brain extract to which had been added 400 ng acetyl-3-methoxytyramine was subjected to this concentrating technique, thus demonstrating its efficiency at the ng level.

Note that the spread on the chromatogram of large amounts (more than 2 μg) of acetyl-dopamine was found to be considerably greater than the spread of the visualized marker. Hence, certain precautions, unnecessary when eluting acetyl-dopamine when present in amounts of the order of 0·6 μg such as occurred on chromatograms of extracts equivalent to 1 g rat whole brain, were required for the quantitative elution of acetyl-dopamine from chromatograms of extracts of tissues such as dog caudate nucleus (Guldberg & Yates, 1969) which contain high concen-

trations of dopamine. Since there was about 6 cm between the front edge of the visualized acetyl-metanephrine and the rear edge of the visualized acetyl-dopamine (Table 2), the spread of acetyl-dopamine in this direction could be readily allowed for by cutting the chromatogram at a distance of 0·5 cm ahead of the front edge of the acetyl-metanephrine marker. But because there was only a 3 cm separation between the acetyl-dopamine and acetyl-3-methoxytyramine markers (Table 2), a significant amount of acetyl-dopamine could be expected in the acetyl-3-methoxytyramine eluates. The acetyl-3-methoxytyramine eluates were therefore assayed for both acetyl-3-methoxytyramine and acetyl-dopamine as follows: A 1·00 ml. portion of the eluate was put through the acetyl-3-methoxytyramine assay technique, which assays both acetyl-3-methoxytyramine and acetyl-dopamine. Standard amounts of acetyl-dopamine (100–400 ng) were processed through the

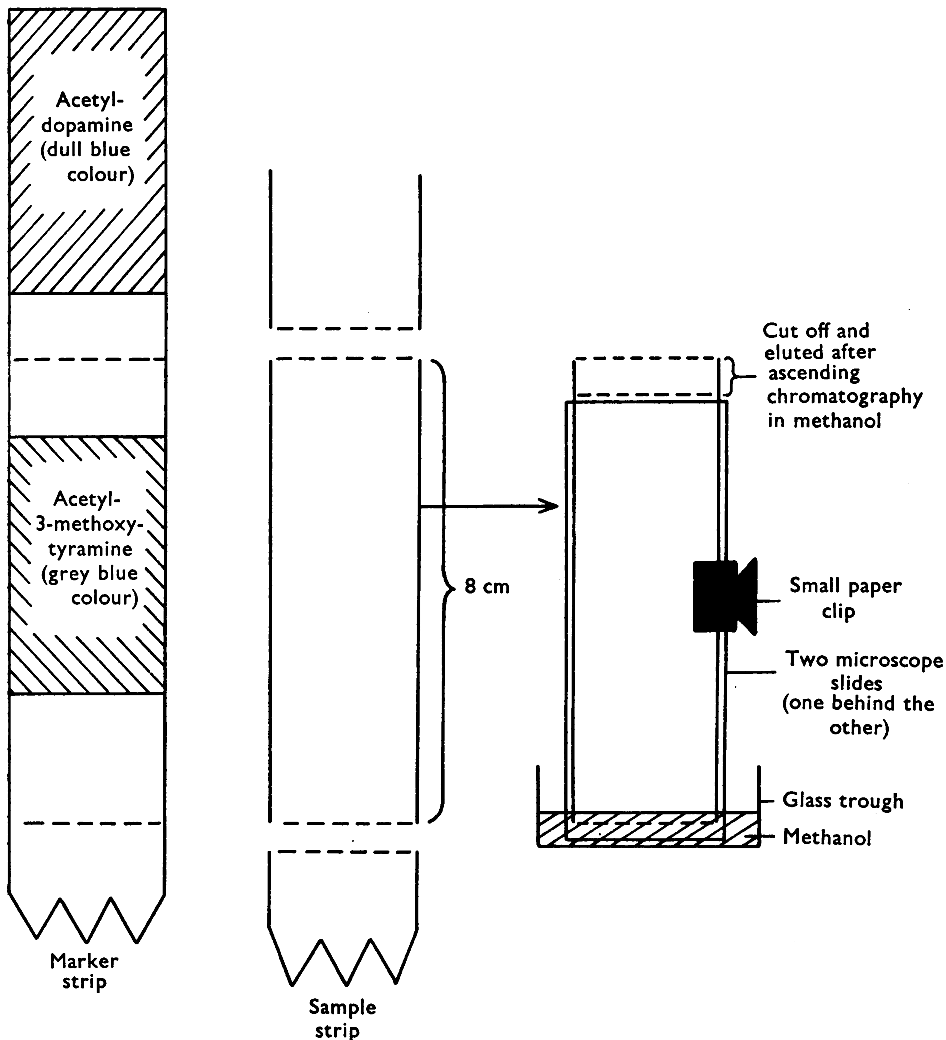

FIG. 2. Technique for concentrating acetyl-3-methoxytyramine spread over 3–5 cm length of a chromatogram strip into a 0·7 cm length of chromatogram strip.

acetyl-3-methoxytyramine assay in order to obtain a measure of the relative fluorescence intensities of these two substances. A second eluate portion, 0·80 ml., was assayed for acetyl-dopamine alone by the acetyl-dopamine assay technique. With this information it was possible to calculate the contribution made by acetyl-dopamine to the fluorescence reading obtained by the acetyl-3-methoxytyramine assay, and hence to calculate the fluorescence due to acetyl-3-methoxytyramine.

Estimation of acetyl-normetanephrine

An adaptation of the method of Brunjes *et al.* (1964) was used. 1·00 ml. 0·4 N hydrochloric acid paper eluate in a 15 ml. glass-stoppered test-tube was heated for 20 min in a boiling water-bath and cooled thoroughly. During the subsequent development of the fluorophor, the sample was kept in the dark by insertion in a solid wooden test-tube rack (Sharman, 1963) because the trihydroxyindole fluorophor derivative of normetanephrine was found to be light sensitive. 0·50 ml. zinc sulphate (0·5% (w/v) $ZnSO_4 \cdot 7H_2O$) in 0·02 N hydrochloric acid was added to the solution followed by 0·50 ml. 0·075% (w/v) potassium ferricyanide (freshly prepared from a 0·25% (w/v) stock solution, stored in a dark bottle at 4° C). Exactly 4 min after the ferricyanide addition the pH of the solution was brought to 5·5 by adding 0·7 ml. 1 M sodium acetate solution previously adjusted to pH 12·9 with 5 N sodium hydroxide. After a further 4 min interval, 0·50 ml. of a freshly prepared mixture containing 8·70 ml. 5 N sodium hydroxide, 0·30 ml. redistilled ethylenediamine and 1·00 ml. freshly prepared 2% (w/v) ascorbic acid, was added. The relative fluorescence was read in the Aminco-Bowman SPF at 400/500 mμ, 20–30 min after addition of the ferricyanide. Reagent blanks and aqueous standards were processed along with each batch of samples. Internal standards were not processed routinely, because their fluorescence intensity was found not to differ significantly from aqueous standards and because, as was also the case in the other assays, it was essential to conserve the available acetylated amine for the estimation in view of the very small amounts present.

Estimation of acetyl-metanephrine

This compound was estimated by the method used to determine acetyl-normetanephrine. Addition of sodium acetate was omitted, since oxidation of hydrolysed acetyl-metanephrine, unlike oxidation of hydrolysed acetyl-normetanephrine, proceeded satisfactorily in 0·4 N hydrochloric acid. The acetyl-metanephrine fluorophor was more rapidly produced and broken down than the acetyl-normetanephrine fluorophor. The fluorescence derived from acetyl-metanephrine, at 430/520 mμ, was therefore measured 15 min after addition of the ferricyanide. Appropriate reagent blanks, aqueous standards and internal standards were also determined.

Extraction and estimation of acetyl-dopa

The following technique was found to be satisfactory for the determination of 0·2–1·0 μg dopa after acetylation in pure solution.

Since acetyl-dopa is extracted into an organic solvent at an acid but not an alkaline pH, the pH of the aqueous phase remaining after acetylation was adjusted to about 2 by the drop-wise addition of concentrated hydrochloric acid. The solution was saturated with sodium chloride and extracted twice with 2 vol.

dichloromethane by shaking for 4 min each time and centrifuging to separate the layers. The combined dichloromethane extracts were stirred with 0·1 vol. 0·1 M pH 8·5 borate buffer and sodium bicarbonate (0·3 g/0·5 ml. acetic anhydride added during the acetylation procedure) until there was no further evolution of carbon dioxide. The sodium bicarbonate was required to neutralize the acetic acid extracted into the dichloromethane from the acidified acetylation reaction mixture. Acetyl-dopa could be determined in this borate buffer extract by the same technique as was used to assay the acetylated catecholamines. The acetyl-dopa fluorophor was not extractable into isobutanol, even after acidification of the solution to a pH of about 1. The fluorescence was therefore measured in the aqueous solution, at 430/540 mμ.

Preliminary experiments have indicated that this technique is suitable for the determination of dopa in extracts of brain tissue prepared as described in this paper. Quenching of the fluorescence from acetyl-dopa by such tissue extracts was negligible. Acetyl-tyrosine, which might be present in the acetyl-dopa fraction from a tissue extract, yielded no detectable fluorescence when tested in 10 μg amounts in the ethylenediamine reaction mixture.

Results

Effect of acetylation on the fluorescence from the amines and dopa

A linear relation was found between the amount of substance present and the fluorescence intensity derived from the acetylated derivatives of adrenaline, noradrenaline, dopamine, 3-methoxytyramine and dopa on applying the appropriate assay method to pure solutions containing 40–600 ng of substance and to acetylnormetanephrine and acetyl-metanephrine on applying the appropriate method to solutions containing 40–200 ng of these derivatives. When 40–200 ng amounts in pure solution were examined, a linear relation was also found for each of the unacetylated amines and for dopa on applying the assay method for the corresponding acetylated compound without the preliminary hydrolysis step.

TABLE 1. *Effect of acetylation on (i) the wavelengths for maximal fluorescence of the fluorophors from the catecholamines, their O-methylated amine metabolites and dopa, and (ii) on the sensitivities of estimation of these amines and dopa*

	Method of assay	Maximal fluorescence. Activation/fluorescence wavelengths (mμ)		Sensitivity of assay in pure soln. (ng)		Sensitivity of assay of acetylated amines in presence of chromatogram eluates (ng)
		Unacetylated	Acetylated	Unacetylated	Acetylated	
Adrenaline	E.D.	430/540	430/500	20	20	30
Noradrenaline	E.D.	430/500	430/500	15	30	60
Dopamine	E.D.	430/525	430/525	45	15	40
3-Methoxytyramine	E.D.	440/515	430/525	50	20	40
Metanephrine	T.H.I.	420/520	420/520	10	10	15
Normetanephrine	T.H.I.	400/500	400/505	20	45	60
Dopa	E.D.	360/470	430/540	20	40	—

Sensitivities calculated as that amount of amine giving the same reading as the blank, at the wavelengths for maximum excitation and emission of the fluorescence. E.D. indicates compound estimated after condensation with ethylenediamine. T.H.I. indicates compound estimated after oxidation with potassium ferricyanide (the " tri-hydroxyindole " method).

The effect of acetylation on the wavelengths for maximum fluorescence and on the sensitivity of assay for each amine and dopa are shown in Table 1. The sensitivity of an assay is assessed, arbitrarily, as that amount of substance which produces a fluorescence reading equal in size to that produced by the appropriate blank at the same wavelengths of excitation and emission.

Acetylation produced a marked change in the wavelengths of maximum fluorescence of fluorophors obtained by condensing ethylenediamine with adrenaline and dopa and a less marked change in the wavelengths of maximum fluorescence from 3-methoxytyramine. The wavelengths of maximum fluorescence of the ethylenediamine-derived fluorophors from noradrenaline and dopamine, or of the fluorophors obtained by oxidizing metanephrine and normetanephrine with potassium ferricyanide, were unaltered by acetylation.

By acetylation, the sensitivities of the assay methods were increased threefold for dopamine and 3-methoxytyramine, reduced by one half for dopa, noradrenaline and normetanephrine and unaltered for adrenaline and metanephrine. The sensitivities of assay of the amines in the presence of eluates from rat brain chromatograms (Table 1) were less than the corresponding sensitivities in pure solution because the paper blanks were up to twice those of the reagent blanks.

The acetylated catecholamines could be assayed by the methods used to estimate their O-methyl derivatives, but the acetylated O-methyl amines could not be assayed by the methods used to estimate the acetylated catecholamines. Fluorophors with the same wavelength characteristics were obtained from an acetylated catecholamine and its respective O-methylated derivative by the method used to estimate the O-methyl amine.

The acetylation reaction

As assessed by fluorimetry, the acetylation technique gave consistent yields of an acetylated amine independent of the amount of the starting material at the ng level.

Separation of the acetylated amines by paper chromatography

Table 2 gives the distances of flow of marker amounts of the acetylated derivatives of the catechol- and O-methyl amines during descending chromatography in the organic phase of the toluene : ethyl acetate : methanol : water mixture. Also included for comparison are the distances of flow of acetyl-octopamine, acetyl-tyramine and acetyl-5-hydroxytryptamine.

TABLE 2. *Distances of flow of the acetylated derivatives of the amines in a typical chromatogram developed with the organic phase of toluene : ethyl acetate : methanol : water* (10 : 1 : 5 : 5 *by vol.*)

Acetylated derivatives of:		Distances of flow from origin (cm)	
Noradrenaline		4·8– 6·0	
	Octopamine		5·4– 6·7
Normetanephrine		6·5– 8·0	
	5-Hydroxytryptamine		14·3–17·0
Adrenaline		18·5–21·3	
Metanephrine		22·3–25·2	
Dopamine		31·7–35·6	
3-Methoxytyramine		39·0–43·0	
	Tyramine		39·5–43·8

Chromatogram (Whatman No. 20 paper) developed in the descending direction for 11 hr at 20°–21° C after equilibration for 9 hr at 20°–21° C.

Concentrations of the amines in whole rat brain

The results from five experiments are given in Table 3. The estimates for rat brain noradrenaline varied from 0·13 to 0·22, with a mean of 0·17 ± 0·03 (S.D.) μg/g. The dopamine estimates varied from 0·55 to 0·76, with a mean of 0·70 ± 0·08 μg/g. Each estimate is the average of analyses from duplicate portions of a perchloric acid brain homogenate. The standard deviation of the difference between duplicates was 0·04 μg/g for noradrenaline and 0·1 μg/g for dopamine. The recoveries of amines added to parallel samples of brain homogenates, unlike the sample estimates, varied widely. The estimates were therefore not corrected for recoveries. An investigation into the reason for these varying and low recoveries is described in a subsequent section.

The fluorescence derived from the " acetyl-dopamine " and " acetyl-noradrenaline " eluates from chromatograms of brain extracts was maximal at the wavelengths of maximum fluorescence of the acetyl-dopamine and acetyl-noradrenaline fluorophors, respectively. The fluorescence intensity of the only two samples containing measurable amounts of normetanephrine were too low to give rise to excitation and emission spectra showing the maxima characteristics of the normetanephrine fluorophor. Five estimates were obtained for 3-methoxytyramine in rat brain ; the fluorescence from which these estimates were calculated was maximal at wavelengths characteristic of the fluorophor derived from authentic acetyl-3-methoxy-tyramine. The wavelengths of maximal fluorescence obtained from eluates from the acetyl-3-methoxytyramine portions of brain chromatograms containing no detectable acetyl-3-methoxytyramine (that is, <0·05 μg 3-methoxytyramine/g brain tissue) were, at 430/500 mμ, the same as those obtained from their paper blanks and were quite different from the characteristics of the acetyl-3-methoxytyramine fluorophor. In none of the rat brain samples were the concentrations of adrenaline and metanephrine above the limit of detection, which was 0·02 μg/g. The limit of detection was taken as that amount of substance giving rise to a fluorescence intensity equal to half that of the blank.

TABLE 3. *Catecholamines and their O-methylated metabolites in rat brain as μg/g tissue (uncorrected for recoveries)*

Experiment	Samples	Noradrenaline	Normetanephrine	Adrenaline	Metanephrine	Dopamine	3-Methoxytyramine
(1)	Duplicates	0·20	<0·05	<0·02	<0·02	0·79	0·05
		0·24	<0·05	<0·02	<0·02	0·74	0·05
(2)	Duplicates	0·14	<0·05	<0·02	<0·02	0·56	<0·05
		0·19	0·04	<0·02	<0·02	0·90	<0·05
(3)	Duplicates	0·17	<0·05	<0·02	<0·02	0·77	0·07
		0·14	<0·05	<0·02	<0·02	0·70	0·10
(4)	Duplicates	0·13	<0·05	<0·02	<0·02	0·64	<0·05
		0·22	<0·05	<0·02	<0·02	0·75	0·10
(5)	Duplicates	0·13	<0·05	<0·02	<0·02	0·48	<0·05
		0·13	0·05	<0·02	<0·02	0·62	<0·05
	Mean	0·17	0·02	0	0	0·70	0·06
S.D. of means of duplicate estimates		0·03				0·08	
S.D. of difference between duplicates		0·04				0·1	

Three rat brains (minus cerebelli) pooled per experiment. Limits of detection (μg/g tissue) calculated on basis that the lowest measurable amount of an amine was that amount giving rise to a fluorescence intensity equal to half that of the blank.

Recoveries of the catechol- and methoxyamines through the procedure

Although the estimates for the endogenous amines were reasonably consistent, there were variable and sometimes marked losses of exogenous amines added to brain homogenates. Control experiments in which the amines in 0·1–1 μg amounts were added at various points in the procedure indicated that a major loss occurred during adjustment of the perchloric acid extract to pH 4. This loss could be decreased if the adjustment was made at 0° C. There were indications that additional variable losses of exogenous amines could occur if exogenous amines were added to a perchloric acid brain homogenate (Table 4).

Discussion

The technique described in this paper enables the catecholamines, their amine and acid metabolites and their amino-acid precursor to be estimated in the same sample of brain tissue. An advantage of this technique is that the amino-acids, the acids and the amines are obtained in three separate fractions. Interference of the components of one fraction with estimation of the components of another fraction is thus eliminated. Although the acid metabolites were not determined in these experiments, the technique for their extraction, as worked out in this paper, proved satisfactory in subsequent experiments using dog brain (Guldberg & Yates, 1969). A method was developed for the estimation of the amino-acid precursor, dopa, which has been used to determine the concentrations of dopa in dog blood and cerebrospinal fluid following an intravenous infusion of dopa (Guldberg & Yates, 1968).

The catecholamines and their O-methylated derivatives were separated from one another in the form of their acetyl derivatives before their estimation. Acetylation of the amines had the following additional advantages: (i) Extraction of the acetylated amines into dichloromethane from the brain extract made their applica-

TABLE 4. *Recoveries of catecholamines and their O-methylated metabolites added to samples of dog caudate nucleus at different stages in the procedure*

	Nor-adrenaline	Adrenaline	Metanephrine	Dopamine	3-Methoxy-tyramine
Amount of amine added:	200 ng	100–200 ng	100–200 ng	1 μg	200–400 ng
Stages of addition of amines to sample:					
1. Just before acetylation	128	110	97	53	68
2. Acid extract just before extraction with ethyl acetate	120	75	92	98	69
3. Extract at pH 4 before $KClO_4$ removed	68	108	59	87	70
4. Extract before adjusting pH to 4, at 0° C (two experiments)	100, 128	88, 62	140, 94	100, 112	136, 44
5. Extract before adjusting pH to 4 (mean±S.D. $n=4$)	52±17	46±11	37±13	81±26	15±10
6. Tissue homogenate (mean±S.D. $n=4$)	40±54	34±31	71±35	21±23	Not done

All additions made at room temperature, unless otherwise indicated. Results are expressed as % of amount added.

tion to a paper chromatogram easy and might explain why the blank from eluates from chromatograms of tissue extracts was no greater than the blank from eluates from chromatograms to which no extract was applied; substances contributing to the blank may not be removed into dichloromethane. (ii) Acetylation makes the catecholamines less readily oxidizable.

Laverty & Sharman (1965) described a method, utilizing acetylation, whereby the concentrations of adrenaline, noradrenaline and dopamine in tissue extracts could be determined. They found that acetylation increased the yield of fluorescence following interaction of ethylenediamine with all three catecholamines, whereas we found that although the fluorescence yield from dopamine was potentiated, the yields from adrenaline and noradrenaline were unaltered and reduced, respectively. Because our fluorescence yield from adrenaline was very much higher than that reported by Laverty & Sharman, this absence of potentiation on acetylation did not make the sensitivity of the acetyl-adrenaline assay any lower than that found by Laverty & Sharman (1965). The sensitivity of acetyl-noradrenaline assay, was, however, lower. No explanation could be found for this discrepancy; it was not due to the fact that Laverty & Sharman (1965) used 2 N hydrochloric acid to prepare their ethylenediamine reagent, whereas we used 4 M ammonium chloride. We developed a new method for the determination of 3-methoxytyramine and its acetylated derivative, whereby a fluorophor was obtained from 3-methoxytyramine by treatment with periodate, which presumably effected a demethylation (Adler & Magnusson, 1959), followed by reaction with ethylenediamine. This method was twice as sensitive as that described by Carlsson & Waldeck (1964). Metanephrine, but not its acetylated derivative, gave a strong fluorescence at 430/540 mμ by this method. The fluorescence yields from normetanephrine and acetyl-normetanephrine by this procedure were, however, poor. Alternative methods of estimation, whereby acetyl-metanephrine and acetyl-normetanephrine were converted to fluorescent lutine derivatives (Brunjes *et al.*, 1964) proved satisfactory. According to Welsh (1955), acetylation of the catecholamines occurs on the phenolic hydroxyls and the amino nitrogen, the formed ester groups being more readily hydrolysed than the amide linkage. Since hydrolysis of substituents on the catechol nucleus is a necessary preliminary to coupling with ethylenediamine (Weil-Malherbe & Bone, 1952) the phenolic acetyl groups cannot contribute to the alteration in fluorescence produced by acetylation. It seems likely that the observed effects of acetylation on the wavelength characteristics and fluorescence yields of fluorophors derived from the catechol- and methoxyamines are due to the persistence of an N-acetyl group in the fluorophors.

In order to obtain quantitative separation of the amines on the paper chromatogram, it is essential to maintain complete acetylation of each of the amines before and during the chromatography. Incomplete acetylation, or partial deacetylation, of an amine gives rise to a mixture of acetylated derivatives which are separated on the chromatogram and in some cases overlap the wholly or partially acetylated derivatives from other amines.

The relative distribution of the acetylated amines on the chromatogram was approximately the same as that reported by Goldstein, Friedhoff, Simmons & Prochoroff (1960), although direct comparison is not possible because these workers developed the chromatogram at 37° C. We found that development at this temperature gave unsatisfactory results due to a temperature gradient across the tank which

gave rise to different flow rates of the acetyl derivatives from one chromatogram strip to another.

Recoveries of the catechol- and methoxyamines through the procedure

Unlike the endogenous amines, the catechol- and methoxyamines added to 0·4 N perchloric acid extracts of brain tissue were subject to variable degrees of loss during the extraction procedure. The results of the control experiments indicated that the losses might occur at two stages in the extraction procedure as a result of (i) adsorption on to the protein precipitate which was subsequently discarded and (ii) destruction in the acid supernatant or during the subsequent adjustment of its pH from 0·8 to 4. Loss of amines added to the 0·4 N perchloric acid extract was prevented if the extract was maintained at about 0° C during addition of the amines and during the subsequent pH adjustment. Because the replicate estimates for rat brain amines and also for dog brain amines (Guldberg & Yates, 1969) were reasonably consistent despite varying recoveries of exogenous amines, it would appear that the endogenous amines must be protected in some way from those influences causing the losses of the exogenous amines. This protection might be afforded by an attachment to a protein or lipid (Barger, 1930; von Euler, 1947), the combination being stable in, and not precipitated by, the perchloric acid. Alternatively, under the extraction conditions described, the endogenous amines may be present in some occluded state. It is, however, difficult to envisage how either of these forms of amine binding would be stable at the relatively low pH of the perchloric acid tissue extracts. The estimate of the concentration of dopamine in the rat brain obtained by the present method is comparable with the values found by other authors. Carlsson (1959) reported a concentration of 0·60 μg/g and Cox & Potkonjak (1967) found a concentration of 0·87 μg/g in this tissue. The present value for the concentration of noradrenaline is lower than that given in these reports and possibly reflects a lower recovery of the endogenous noradrenaline.

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