Brit. J. Pharmacol. (1965), **24**, 538–548.

THE ESTIMATION OF SMALL QUANTITIES OF 3,4-DIHYDROXYPHENYLETHYLAMINE IN TISSUES

BY

R. LAVERTY* AND D. F. SHARMAN

From the Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge

(*Received September* 18, 1964)

Several methods have been used to estimate small amounts of 3,4-dihydroxyphenylethylamine (dopamine) which occur in many plant and animal tissues. The biological assay of dopamine is, by comparison with that of other catechol amines, relatively insensitive. The pressor effect of dopamine on the blood pressure of the rat and the depressor effect observed on the blood pressure of the guinea-pig have been used to assay dopamine extracted from the splenic nerve and stellate ganglion of the ox (Schümann, 1956). These methods are suitable for microgram quantities of dopamine.

Of the chemical methods which are applicable to the estimation of dopamine only those involving the formation of a fluorescent derivative are likely to be sensitive enough to estimate the small amounts of dopamine occurring in some tissues. The main fluorimetric methods are based on two chemical reactions. The first of these was described by Natelson, Lugovoy & Pincus (1949) who showed that catechol amines could condense with ethylenediamine to yield intensely fluorescent compounds. This reaction, which depends on the presence of the catechol group, was developed by Weil-Malherbe & Bone (1957) and used to estimate catechol amines including dopamine. The reaction was also used by Euler & Lishajko (1957) to identify and estimate the dopamine in beef splenic nerve. The major criticism of this method lies in its lack of specificity. The second reaction which has been used involves the conversion of dopamine into an indole derivative by oxidation and treatment with alkali, which then shows a fluorescence in acid solution (Carlsson & Waldeck, 1958). This method has been used with slight modifications by several authors (Drujan, Sourkes, Layne & Murphy, 1959; McGeer & McGeer, 1962) and has the advantage of a greater specificity. In this laboratory the latter method was found to be unworkable, apparently because of the presence of material in the water supply which is not easily removed by distillation. We have therefore been compelled to use the condensation with ethylenediamine as the basis for the estimation of dopamine. This paper describes ways in which the specificity and sensitivity of this method can be improved so that very small quantities of dopamine may be extracted from tissues, identified with reasonable certainty and estimated.

METHODS

Materials. These were ascorbic acid (Roche Products), adrenaline base (Burroughs Wellcome), noradrenaline bitartrate (L. Light), dopamine hydrochloride (California Foundation for Biochemical Research),

* Beit Memorial Research Fellow. Present address: Wellcome Research Institute, University of Otago Medical School, Dunedin, New Zealand.

isoprenaline sulphate (Boots), 6-hydroxydopamine hydrobromide (Merck Sharpe & Dohme), catechol (B.D.H.), α-methyldopamine hydrochloride, and ethylenediamine (May & Baker) distilled at least three times until reagent blank fluorescence was low and constant, dichloromethane (G.P.R.; Hopkin & Williams) distilled once, isobutanol (A.R.) distilled three times, acetic anhydride (A.R.; B.D.H.), sodium chloride (R grade; May & Baker) check reagent blank fluorescence with each new batch distilled three times, and hydrochloric acid (M.A.R.; Hopkin & Williams).

Methanol was purified by distilling from sodium hydroxide and then redistilled. Chromatography solvents were of analytical reagent grade and distilled once before use. All other chemicals were of Analytical Reagent grade.

Other materials were Dowex 50WX-8 cation exchange resin (Dow Chemical Co.), Whatman No. 50 paper for chromatography (extracted with 2 N-sodium hydroxide solution and washed as described by Sharman, 1963), and deionized and distilled water.

The acetylation and extraction of dopamine were based on the methods described by Hagopian, Dorfman & Gut (1961) for the extraction of dopamine and its metabolites from biological media and applied to the estimation of catechol amines in urine by Goldstein, Friedhoff & Simmons (1959).

The preparation of tissue extracts for acetylation. Peripheral nervous tissues and spinal cords were frozen in liquid nitrogen and crushed in a stainless steel instrument similar to a tablet die. The powdered tissue was then homogenized in 0.1 N-hydrochloric acid or in 0.1 N-sulphuric acid in a glass homogenizer. Other central nervous tissues were homogenized directly in the acid. In each instance a few mg of ascorbic acid were added to the acid before homogenizing the tissues. If a direct extraction from a deproteinized solution was to be made, sulphuric acid was used and this also served as the protein precipitant. The homogenates prepared in sulphuric acid were left for 15 min at 4° C, centrifuged at 6,000 *g* for 5 min at 0° C and the supernatant fluid was acetylated. In those experiments in which the catechol amines were adsorbed on to a column of Dowex 50WX-8 cation exchange resin, the tissue was homogenized in hydrochloric acid. The proteins were then precipitated by adding perchloric acid to make the solution 0.4 N. Adsorption of the catechol amines onto the resin was carried out essentially as described by Bertler, Carlsson & Rosengren (1958). The total catechol amines were eluted from the column with 8 to 10 ml. of 2 N- or 3 N-hydrochloric acid. Disodium edetate (20 μg) was added to the eluate which was then partially neutralized by the addition of approximately 1.2 g of sodium bicarbonate. The eluates were then ready for acetylation. A reasonable separation of adrenaline and noradrenaline from dopamine could be obtained by eluting the former two amines from the column with 10 ml. of 0.4 N-hydrochloric acid and the latter amine with 8 ml. of 2 N-hydrochloric acid. Two compounds closely related to dopamine, 6-hydroxydopamine and α-methyldopamine, were found to be eluted mainly in the second fraction.

Acetylation of solutions containing catechol amines. Acetic anhydride was added to the solution in the proportion of 0.3 ml. of the anhydride for each 4 ml. of solution. A slight excess of powdered sodium bicarbonate was then added in small portions and the solution was shaken as soon as the effervescence subsided. The mixture was ready for extraction when all the acetic anhydride had disappeared. The procedure took about 15 min. Details of the products formed with some catechol amines, usually 3-*O*,4-*O*,*N*-triacetyl derivatives, are described by Welsh (1955).

Extraction of the acetylated catechol amines. After acetylation the solution was extracted twice with two times its volume of dichloromethane. The dichloromethane extracts were filtered through anhydrous sodium sulphate, and evaporated to approximately 0.2 ml. under a stream of air in a water bath at 40 to 50° C.

Paper chromatography. All chromatograms were developed on alkali-washed Whatman No. 50 paper. This paper shows a minimum of background fluorescence. The sheets of paper were cut into lanes so that each sample was run on a 2-cm-wide strip. Two solvent systems have been used: a slight modification of Bush System C (Bush, 1952) consisting of toluene, ethyl acetate, methanol and water in the proportions 10 : 1 : 5 : 5 by volume. The chromatogram was equilibrated with the aqueous phase for 16 hr and then developed for 4 to 5 hr with the organic phase.

When a separation of 6-hydroxydopamine (a possible metabolite of dopamine; Senoh, Creveling, Udenfriend & Witkop, 1959) from dopamine was required a solvent system based on one of the *tert.*-butanol systems described by Eberlein & Bongiovanni (1955) was found to be suitable. This was a mixture of petroleum spirit (boiling point 80 to 100° C), *tert.*-butanol and water in the proportions 8 : 3 : 8 by volume.

The chromatogram was equilibrated with the aqueous phase for 16 hr and then developed for approximately 18 hr with the organic phase.

The acetylated derivatives of the catechol amines were eluted from small portions of the chromatograms by shaking the piece of paper with 4 ml. of water for 1 hr. The paper was removed before the condensation with ethylenediamine was carried out.

Acetyl derivatives prepared from authentic catechol amines were run on marker strips in parallel with each set of estimations. The marker strips were sprayed with a fivefold dilution of the ethylenediamine–hydrochloric acid mixture described in the following section, placed between two sheets of glass and heated at 80° C for 20 min. The position of the acetyl derivatives of the catechol amines on the chromatogram could be easily seen by the coloured, fluorescing spots which developed. Usually the position of the dopamine derivative was determined and that portion of each chromatogram strip lying between the origin and a point 2 cm in front of the dopamine position was divided into eight or ten equal portions. Each of these was eluted and condensed with ethylenediamine. Measurement of fluorescence yielded a profile of the catechol derivatives present on the chromatogram. In a few experiments the whole length of the chromatogram was divided up to see if other substances giving rise to a fluorescent derivative could be detected.

Condensation with ethylenediamine. A mixture of ethylenediamine and 2 N-hydrochloric acid, 3 : 2 by volume, was freshly prepared. Of this solution 0.5 ml. was added to the 4.0 ml. of eluate from the chromatogram and mixed carefully. The mixture was then heated at 60 to 65° C for 20 min in the dark. The samples were removed from the water bath, cooled, saturated with sodium chloride and then shaken with 3.0 ml. of isobutanol for 4 min. After centrifugation, the isobutanol layer was examined fluorimetrically. Standards were prepared by acetylating known amounts of catechol amines in a volume of 4 ml. and condensing them with ethylenediamine. This latter technique of acetylation and condensation without extracting the acetylated derivative can also be applied to the dopamine eluate from resin columns.

Fluorescence. Activation and fluorescence spectra were determined in an Aminco-Bowman spectrophotofluorimeter. Wavelengths given in this paper are uncorrected instrumental values. Calibration against a mercury line spectrum indicated an accuracy of ± 5 mμ in the range of the wavelengths of maximum activation and fluorescence. Routine estimations were made on a Locarte filter fluorimeter modified to take square quartz cuvettes ($1.0 \times 1.0 \times 4.4$ cm; internal measurements), and fitted with a device to enable the rapid changing of filters in the fluorescent light path. In a few experiments the volumes used were one-fifth of those described above and the fluorescence was measured in a round cuvette (0.4 cm diameter $\times$ 3.8 cm length; internal measurements). This resulted in a further fourfold increase in the sensitivity of the method.

The primary (activation) filter was a combination of a Corning 3389 filter and a Corning 5113 (half standard thickness) filter with the former filter nearest to the mercury lamp. This set transmits mainly the line of the mercury spectrum at 436 mμ. Two secondary (fluorescence) filters were used so that differential fluorimetry, based on differences in fluorescence spectra, could be carried out. These were Ilford Bright-Spectrum No. 623 (maximum transmission 490 mμ) and Ilford Bright-Spectrum No. 625 (maximum transmission 540 mμ).

The differential fluorimetric estimation of mixtures of dopamine and another catechol amine (called C) was carried out using the following equations. C represents a catechol amine which gives a fluorescence spectrum different from that given by dopamine.

$$\mu\text{g of dopamine} = (BC_1 - AC_2)/(C_1D_2 - C_2D_1)$$

$$\mu\text{g of C} = [A - (\mu\text{g dopamine} \times D_1)]/C_1$$

where A = fluorescence reading with filter 1

B = fluorescence reading with filter 2

D_1 = fluorescence for 1.0 μg of dopamine on filter 1

D_2 = fluorescence for 1.0 μg of dopamine on filter 2

C_1 = fluorescence for 1.0 μg of other catechol amine with filter 1

C_2 = fluorescence for 1.0 μg of other catechol amine with filter 2

RESULTS

The effect of acetylation on the fluorescence obtained by condensing catechol amines with ethylenediamine. If catechol amines are acetylated before condensing them with ethylenediamine the intensity of the fluorescence developed is increased. Furthermore, the wavelength of maximal fluorescence may be changed. Condensates derived from adrenaline and from 6-hydroxydopamine fluoresce at a shorter wavelength. These changes are shown in Table 1, which also shows that after acetylation dopamine and α-methyldopamine can now be distinguished from those catechol derivatives which before acetylation gave rise to almost the same fluorescence spectrum.

The separation of the acetyl derivatives of catechol amines on paper chromatograms. The R_F values of some acetylated catechol amines in the modified Bush system C are given in Table 2. The R_F values obtained for the first three compounds are in agreement with the results of Goldstein, Friedhoff, Simmons & Prochoroff (1959). Table 2 also shows that the derivatives of dopamine and α-methyldopamine are separated in the modified Bush solvent system C. Fig. 1 illustrates the separation of acetylated 6-hydroxydopamine from the dopamine derivative in the *tert.*-butanol solvent system.

Recovery of catechol amines. Small quantities of catechol amines were added to tissue homogenates and estimated after acetylation using the direct extraction method described above. Table 3 shows the recovery of noradrenaline and dopamine from tissue homogenates. Dopamine appears to be well recovered from tissue homogenates, but noradrenaline shows a variable and somewhat lower recovery. This difference might be explained by the proximity of the noradrenaline acetate position to the origin of the chromatogram so that other substances might be included in the eluate which cause a reduction in the fluorescence developed from the noradrenaline.

When the resin column stage, which involves a loss of approximately 35%, is included in the extraction procedure the expected overall recovery would be about 40%. In two experiments in which dopamine was recovered from tissue homogenates by this procedure the recovery was 44 and 32%. The estimation of adrenaline has not yet been examined in detail.

Application to nervous tissue. In a preliminary survey the methods have been used to make a few or single observations on a wide range of nervous tissues from different species so that the range and applicability of the methods could be examined.

A combination of the resin column extraction, acetylation and paper chromatographic separation of acetylated catechol amines was applied to a selection of nervous tissues obtained from dogs and cats and the dopamine and noradrenaline contents were estimated. The results obtained are given in Table 4. They agree with previous observations on the distribution of dopamine in nervous tissues (Bertler & Rosengren, 1959) and also show the presence of dopamine in sympathetic ganglia other than the stellate ganglion (Schümann, 1956).

Figs. 1 and 2 show the fluorescence profiles of that part of chromatograms of extracts of dog hypothalamus and superior cervical ganglion where the acetylated naturally occurring catechol amines are found. They were developed with the *tert.*-butanol solvent system and show well-defined dopamine peaks. There was, however, a very small amount of fluorescence detected about 18 cm in front of the dopamine region. This has not been

TABLE 1

THE ACTIVATION AND FLUORESCENCE MAXIMA AND THE RELATIVE FLUORESCENCE INTENSITIES OF THE PRODUCTS OBTAINED AFTER CONDENSATION OF CATECHOL AMINES AND THEIR ACETYLATED DERIVATIVES WITH ETHYLENE DIAMINE

The maxima are given for the aqueous solution after condensation and for the isobutanol extract thereof; the relative intensity is for 1 μg of catechol amine in 4 ml. of water and 3 ml. of isobutanol at its maximum wavelengths (uncorrected instrumental values)

	Direct condensation						Condensation after acetylation					
	Wavelength maxima in water			Wavelength maxima in isobutanol			Wavelength maxima in water			Wavelength maxima in isobutanol		
Catechol compound	Activation (mμ)	Fluorescence (mμ)	Relative fluorescence intensity	Activation (mμ)	Fluorescence (mμ)	Relative fluorescence intensity	Activation (mμ)	Fluorescence (mμ)	Relative fluorescence intensity	Activation (mμ)	Fluorescence (mμ)	Relative fluorescence intensity
Noradrenaline	410	510	153	420	490	960	410	510	220	420	490	1,680
Adrenaline	410	530	27	420	525	250	410	510	196	420	490	1,510
Dopamine	410	540	28	420	525	250	410	540	78	430	520	980
α-Methyldopamine	410	540	25	420	525	210	410	540	52	430	520	680
6-Hydroxydopamine	410	510	28	420	520	280	410	510	72	415	485	630
Isoprenaline	410	510	16·5	420	500	87	410	510	67	420	490	496
3,4-Dihydroxyphenylacetic acid	410	530	87	420	490	230	410	530	85	420	490	270
Catechol	410	510	380	420	490	2,760	410	510	420	420	490	3,000

TABLE 2

R_F VALUES OF ACETYLATED CATECHOL DERIVATIVES IN BUSH SOLVENT SYSTEM C

Acetyl derivative of	R_F
Noradrenaline	0·05
Adrenaline	0·19
Dopamine	0·33
6-Hydroxydopamine	0·35
α-Methyldopamine	0·45
Isoprenaline	0·63
Catechol	0·90

TABLE 3

RECOVERY OF CATECHOL AMINES ADDED TO TISSUE HOMOGENATES

Amount added (ng)	Amount recovered	
	Dopamine (ng)	Noradrenaline (ng)
100	85	58
100	66	69
100	77	46
100	74	58
100	85	50
50	37	31
50	28	17
25	17·5	25
25	12	6

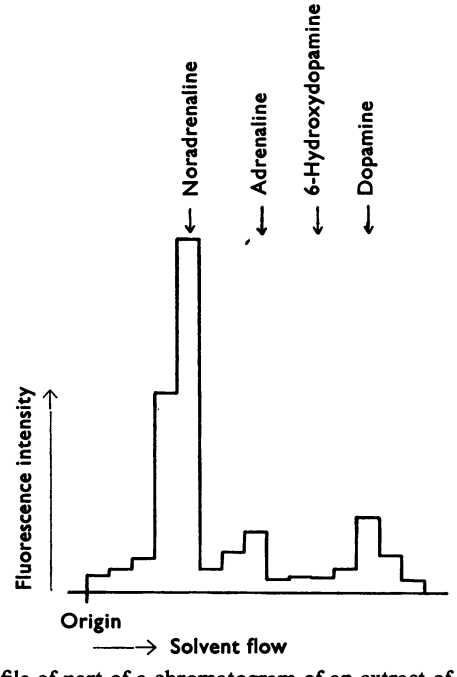

Fig. 1. Fluorescence profile of part of a chromatogram of an extract of dog hypothalamus. Developing solvent—petroleum spirit : *tert.*-butanol : water. Arrows show position of acetyl derivatives of authentic catechol amines.

TABLE 4

THE CONCENTRATION OF NORADRENALINE AND DOPAMINE IN NERVOUS TISSUES

The extraction method included a cation exchange resin column and the results were corrected for a recovery of 40%. Tissues were pooled samples from four cats and three dogs. * These estimations were made on the thoracic and lumbar cord from each of two cats and on a pooled sample of portions of the thoracic cord from two dogs. Concentrations refer to fresh tissue

	Cat			Dog		
	Weight of tissue	Concentration		Weight of tissue	Concentration	
Tissue	(g)	Noradrenaline (μg/g)	Dopamine (μg/g)	(g)	Noradrenaline (μg/g)	Dopamin (μg/g)
Caudate nucleus	1·71	0·10	9·9	1·64	0·09	9·9
Hypothalamus	0·73	2·85	0·20	0·95	1·35	0·25
Massa intermedia of thalamus	1·19	0·67	0·05	2·10	0·17	0·05
Midbrain	2·22	0·58	0·15	2·54	0·43	0·33
Cerebral cortex	1·25	0·12	0·07	1·70	0·16	0·01
Palaeocerebellum	1·21	0·12	<0.003	1·61	0·05	0·003
Spinal cord*	1·91	0·16	0·007	1·00	0·10	0·008
	2·89	0·08	0·010			
Splenic nerve		—	—	0·26	12·2	0·45
Cardiac nerve		—	—	0·07	7·8	0·78
Sympathetic chain		—	—	0·65	2·2	0·17
Superior cervical ganglion		—	—	0·35	26·5	2·00
Inferior cervical ganglion		—	—	0·20	5·5	0·80
Stellate ganglion		—	—	0·58	15·9	1·53

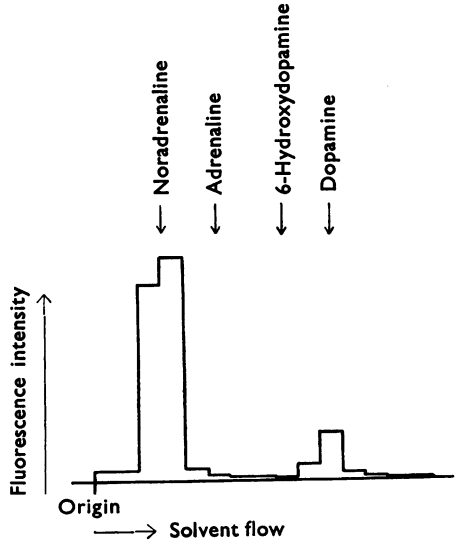

Fig. 2. Fluorescence profile of part of a chromatogram of an extract of dog superior cervical ganglion. Developing solvent—petroleum spirit : *tert.*-butanol : water. Arrows show the position of acetyl derivatives of authentic catechol amines.

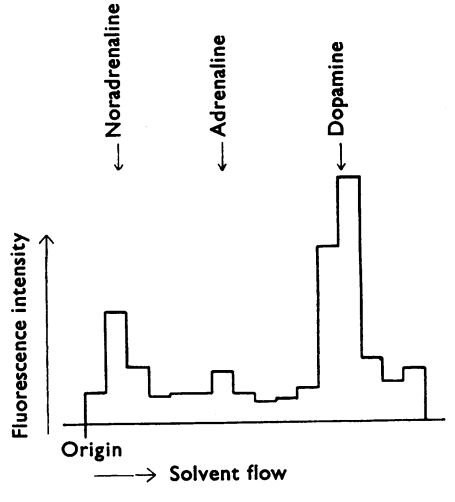

Fig. 3. Fluorescence profile of part of a chromatogram of an extract of the median eminence and pituitary stalk of the cat. Developing solvent—toluene : ethyl acetate : methanol : water. Arrows show position of acetyl derivatives of authentic catechol amines.

identified. The results obtained from experiments in which the acetylated catechol amines were extracted directly from deproteinized solutions are given in Table 5. They demonstrate the presence of dopamine in the superior cervical ganglion of several species and also show

that dopamine is a major catechol amine in the pituitary stalk and median eminence, a result which has been suggested by the histological and pharmacological examination of this tissue by Fuxe (1964).

The estimation of the dopamine in a single pituitary stalk and median eminence of the cat is at the lower limit of the sensitivity of the method. By using the tissue from two cats and employing the microcell it is possible to find not only dopamine, but also adrenaline and noradrenaline in the pituitary stalk and median eminence of the cat. A profile of the fluorescence from such a chromatogram is given in Fig. 3 and shows the presence of the three catechol amines in this tissue.

TABLE 5

THE CONCENTRATION OF NORADRENALINE AND DOPAMINE IN NERVOUS TISSUES

Results were obtained by extracting the acetylated amines directly from deproteinized tissue homogenates. Number of animals from which pooled tissue sample was obtained are given in parentheses. Concentrations are in μg/g of fresh tissue corrected for a mean recovery of 71% for dopamine and 56% for noradrenaline. * Not estimated

Species	Weight (mg)	Tissue	Noradrenaline	Dopamine
Rat	132	Caudate nucleus (3)	0·27	6·39
	116	Hypothalamus (3)	1·29	0·14
	199	Midbrain (3)	0·55	0·13
	165	Cerebral cortex (3)	0·18	<0.01
Rabbit	87	Hypothalamus (1)	1·52	0·20
	610	Midbrain (2)	0·59	0·18
	88	Massa intermedia of thalamus (1)	0·36	0·10
	10	Stellate ganglion (1)	13·0	4·64
	25	Superior cervical ganglion (1)	3·5	0·85
Cat	69	Hypothalamus (1)	2·43	0·20
	78	Superior hypothalamus (2)	1·23	0·35
	108	Medial hypothalamus (2)	2·59	0·25
	52	Inferior hypothalamus (2)	2·89	0·81
	102	Globus pallidus and putamen (1)	0·05	8·22
	34	Superior cervical ganglion (1)	5·25	0·82
	44	Stellate ganglion (1)	2·82	1·32
	7	Pituitary stalk and median eminence (1)	1·1	1·3
	20	Pituitary stalk and median eminence (2)	2·9	8·7
Sheep	81	Caudate nucleus (1)	*	11·78
	178	Superior hypothalamus (1)	3·01	0·27
	218	Inferior hypothalamus (1)	1·44	0·21
	256	Massa intermedia of thalamus (1)	0·70	0·34
	36	Pituitary stalk and median eminence (1)	0·32	5·05
	400	Stellate ganglion (1)	4·07	1·48
Goat	156	Caudate nucleus (1)	*	10·9
	253	Superior hypothalamus (1)	1·48	0·15
	222	Medial hypothalamus (1)	1·81	0·10
	85	Inferior hypothalamus (1)	2·16	0·11
	244	Massa intermedia of thalamus, medial (1)	0·34	0·31
	283	Massa intermedia of thalamus, lateral (1)	0·12	0·46
	445	Anterior midbrain (1)	0·29	0·41
	342	Medial anterior midbrain (1)	0·45	0·27
	302	Medial posterior midbrain (1)	0·55	0·14
	425	Posterior midbrain (1)	0·34	0·04
	39	Pituitary stalk and median eminence (1)	0·16	2·0
	165	Stellate ganglion (1)	1·95	0·85

DISCUSSION

The method described here for the detection, identification and estimation of small quantities of dopamine has overcome some of the difficulties of the earlier methods in which ethylenediamine was used to estimate catechol amines. It is possible to identify as little as 10 to 20 ng of dopamine in tissue. The specificity of the fluorescence developed with dopamine has been improved by acetylating the catechol amines before condensing them with ethylenediamine and by determining the position of the acetates on a paper chromatogram. Of the catechol amines normally present in tissues only dopamine will give rise to a product having a maximum fluorescence at 520 mμ. α-Methyldopamine was the only other catechol compound of those investigated which gave rise to a similar fluorescence. The acetyl derivative of this substance was separated from the dopamine triacetate by paper chromatography and thus, if present, would not interfere with the dopamine estimations. 3,4-Dihydroxyphenylalanine (dopa) and 3,4-dihydroxyphenylacetic acid were not extracted by the procedures used for the catechol amines. A small amount of fluorescence was obtained when 3,4-dihydroxymandelic acid was acetylated, extracted with dichloromethane and then condensed with ethylenediamine. This might be due to the presence of 3,4-dihydroxybenzaldehyde, formed by oxidation, in the sample of acid used. 3,4-Dihydroxybenzaldehyde, 3,4-dihydroxyphenylethanol and 3,4-dihydroxyphenylglycol, possible catechol intermediates in the metabolism of catechol amines, have not yet been examined, but they would not be included in the eluates from a cation exchange resin column. Under normal conditions these latter substances might be expected to be present in tissues in very small quantities only, but must be considered when processes altering the metabolism of catechol amines are being investigated.

One disadvantage of the present method when the resin column stage is omitted, is its inability to distinguish between dopamine and *N*-acetyldopamine, a substance which can be formed *in vivo* (Goldstein & Musacchio, 1962) and also between other catechol amines and their *N*-acetyl derivatives. However, the results of Goldstein (1964) appear to indicate that *N*-acetyl derivatives of dopamine and its metabolites do not occur in the brain tissue of the rat after administration of dopa. Thus it can be concluded that the method can be used to identify and estimate small quantities of dopamine with reasonable certainty and accuracy. The estimation of noradrenaline by the method as it stands is not as good as that of dopamine. This is probably explained by the proximity of the noradrenaline acetate region to the origin of the chromatogram and resultant interference with the development of the fluorescence by material left at the origin. This could probably be overcome by developing the chromatogram for a longer time. A further difficulty lies in the estimation of noradrenaline in the presence of a large amount of dopamine. The acetylation of dopamine resulted in the formation of about 99.5% of the 3,*O*,4-*O*,*N*-triacetyl derivative and there was about 0.5% of an incompletely acetylated derivative present on the chromatogram which runs to the same place as the noradrenaline triacetate. The two substances can be estimated by differential fluorimetry but a small amount of noradrenaline in the presence of a large amount of dopamine can be easily overlooked. This fact also limits the use of the method for radioactive estimations.

The methods described here are applicable to the estimation of small quantities of dopamine in tissues. When larger amounts of tissues (0.5 to 5.0 g) are involved it is advisable to use the resin column stage in the extraction, although the recovery of dopamine is rather

low. For quantities of tissues below 0.5 g this stage can be omitted with a concomitant increase in the recovery of dopamine, but the observations made above with regard to other catechol derivatives must be taken into account.

The amount of dopamine estimated in various parts of the nervous system is, in general, in agreement with that found by other workers (Bertler & Rosengren, 1959). However, only very small quantities of dopamine have been detected in spinal cord, a result which differs from the observations of McGeer & McGeer (1962) who reported a concentration of 0.45 μg/g in the spinal cord of the cat. Observations made in this laboratory have shown that the concentration of dopamine in tissues can be extremely variable and it is certainly advisable to examine the spinal cord more fully. According to Hamberger, Norberg & Sjöqvist (1964), Holmstedt and his colleagues have been unable to detect dopamine in significant amounts in the superior cervical ganglion of the cat. The present experiments clearly demonstrate the presence of this amine in all the sympathetic ganglia which were examined and indicate the degree of sensitivity of the method. This is further illustrated by the demonstration that the major catechol amine in the median eminence and pituitary stalk of the cat, sheep and goat is dopamine.

SUMMARY

1. Because of difficulties encountered in the estimation of small amounts of dopamine, a method has been developed that enables the identification and estimation of as little as 10 to 20 ng of this substance.

2. Dopamine and α-methyldopamine can be distinguished from a number of other catechol derivatives by first acetylating these substances with acetic anhydride in the presence of sodium bicarbonate and then condensing the acetyl derivatives with ethylenediamine. The condensates formed from dopamine and α-methyldopamine fluoresce at a longer wavelength than the condensates derived from the other compounds. The intensity of the fluorescence developed is also increased by acetylating the catechol amines before condensing them with ethylenediamine.

3. Acetylated catechol amines are easily extracted from aqueous solutions into dichloromethane.

4. The acetyl derivatives of catechol amines can be separated by paper chromatography. Two solvent systems used were toluene, ethyl acetate, methanol and water (10 : 1 : 5 : 5), and petroleum spirit, *tert.*-butanol and water (8 : 3 : 8). The former system separated acetylated dopamine from acetylated α-methyldopamine. The latter system was required to separate acetylated dopamine from acetylated 6-hydroxydopamine.

5. The catechol amines in extracts prepared from nervous tissues were acetylated, extracted and then separated by paper chromatography. They were then estimated by differential fluorimetry after condensing the acetyl derivatives with ethylenediamine. Dopamine was present in the superior cervical ganglia of the dog, the cat and the rabbit. The major catechol amine in the pituitary stalk and median eminence of the cat, the goat and the sheep was dopamine.

We would like to thank Dr M. Vogt, F.R.S., for her advice and encouragement. We are grateful to Dr C. S. Miller (Merck Sharp & Dohme Research Laboratory, Rahway, N.J., U.S.A.) for a gift of 6-hydroxydopamine and to Dr S. E. Smith (St. Thomas's Hospital, London) for a sample of α-methyldopamine.

REFERENCES

BERTLER, Å., CARLSSON, A. & ROSENGREN, E. (1958). A method for the fluorimetric determination of adrenaline and noradrenaline in tissues. *Acta physiol. scand.*, **44**, 273–292.

BERTLER, Å. & ROSENGREN, E. (1959). Occurrence and distribution of catecholamines in brain. *Acta physiol. scand.*, **47**, 350–361.

BUSH, I. E. (1952). Methods of paper chromatography of steroids applicable to the study of steroids in mammalian blood and tissues. *Biochem. J.*, **50**, 370–378.

CARLSSON, A. & WALDECK, B. (1958). A fluorimetric method for the determination of dopamine (3-hydroxytyramine). *Acta physiol. scand.*, **44**, 293–298.

DRUJAN, B. D., SOURKES, T. L., LAYNE, D. S. & MURPHY, G. F. (1959). The differential determination of catecholamines in urine. *Canad. J. Biochem.*, **37**, 1153–1159.

EBERLEIN, W. & BONGIOVANNI, A. M. (1955). New solvent systems for the resolution of corticosteroids by paper chromatography. *Arch. Biochem.*, **59**, 90–96.

EULER, U. S. VON & LISHAJKO, F. (1957). Dopamine in mammalian lung and spleen. *Acta physiol. pharmacol. neerl.*, **6**, 295–303.

FUXE, K. (1964). Cellular localisation of monoamines in the median eminence and infundibular stem of some mammals. *Z. Zellforsch.*, **61**, 710–724.

GOLDSTEIN, M. (1964). Cerebral metabolism of DOPA-C^{14} in rats. *Int. J. Neuropharmacol.*, **3**, 37–43.

GOLDSTEIN, M., FRIEDHOFF, A. J. & SIMMONS, C. (1959). A method for the separation and estimation of catechol amines in urine. *Experientia (Basel)*, **15**, 80–81.

GOLDSTEIN, M., FRIEDHOFF, A. J., SIMMONS, C. & PROCHOROFF, N. N. (1959). The metabolism of 3-hydroxytyramine-1-C^{14} in brain tissue homogenates. *Experientia (Basel)*, **15**, 254–256.

GOLDSTEIN, M. & MUSACCHIO, J. M. (1962). The formation *in vivo* of N-acetyldopamine and N-acetyl-3-methoxydopamine. *Biochim. biophys. Acta (Amst.)*, **58**, 607–608.

HAGOPIAN, M., DORFMAN, R. I. & GUT, M. (1961). A method for the isolation and separation of catecholamines and their transformation products from biological media. *Analyt. Biochem.*, **2**, 387–390.

HAMBERGER, B., NORBERG, K.-A. & SJÖQVIST, F. (1964). Evidence for adrenergic nerve terminals and synapses in sympathetic ganglia. *Int. J. Neuropharmacol.*, **2**, 279–282.

MCGEER, E. G. & MCGEER, P. L. (1962). Catecholamine content of spinal cord. *Canad. J. Biochem.*, **40**, 1141–1151.

NATELSON, S., LUGOVOY, J. K. & PINCUS, J. B. (1949). A new fluorometric method for the determination of epinephrine. *Arch. Biochem.*, **23**, 157–158.

SCHÜMANN, H. J. (1956). Nachweis von Oxytyramin (Dopamin) in sympathischen Nerven und Ganglien. *Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak.*, **227**, 566–573.

SENOH, S., CREVELING, C. R., UDENFRIEND, S. & WITKOP, B. (1959). Chemical, enzymatic and metabolic studies on the mechanism of oxidation of dopamine. *J. Amer. chem. Soc.*, **81**, 6236–6240.

SHARMAN, D. F. (1963). A fluorimetric method for the estimation of 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid) and its identification in brain tissue. *Brit. J. Pharmacol.*, **20**, 204–213.

WEIL-MALHERBE, H. & BONE, A. D. (1957). The estimations of catecholamines in urine by a chemical method. *J. clin. Path.*, **10**, 138–147.

WELSH, L. H. (1955). The analysis of solutions of epinephrine and norepinephrine. *J. Amer. pharm. Ass.*, **44**, 507–514.