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THE QUANTITATIVE SEPARATION OF ADRENALINE AND *NOR*ADRENALINE IN BIOLOGICAL FLUIDS AND TISSUE EXTRACTS

BY

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The qualitative similarity in the biological effects and chemical reactions of adrenaline and *nor*adrenaline has made difficult the determination of these substances when present together in biological fluids or tissue extracts. Several methods, however, have been devised. For the purpose of review these methods may be conveniently divided into two groups, pharmacological and chemical.

The pharmacological methods are based on the quantitative difference in the activities of the two amines on certain pharmacological preparations. In a number of such procedures parallel quantitative assays are carried out on at least two preparations: one of these is much more sensitive to adrenaline than to *nor*adrenaline, e.g., the isolated uterus of the non-pregnant rat or the isolated rectal caecum of the hen or chick; whereas the other is more sensitive to *nor*adrenaline or has the same sensitivity to both amines, e.g., the isolated colon of the rat, the perfused ear of the rabbit, the cat's blood pressure, or the denervated nictitating membrane of the cat (Bergström, Euler, and Hamberg, 1950; Bülbring, 1949; Gaddum and Lembeck, 1949; Gaddum, Peart, and Vogt, 1949; Mann and West, 1950). By the use of mathematical formulae incorporating the results of the assays in terms of one or other of the two amines and the activity ratio of the two amines on each preparation the amount of adrenaline and of *nor*adrenaline in the mixture may be determined.

It will be evident that the estimates of adrenaline and *nor*adrenaline are interdependent. Any inaccuracy in the assays or in the comparison of the activities of the amines will be reflected in the values for both adrenaline and *nor*adrenaline. In addition the chance of inaccuracy might be expected to increase when biological fluids or tissue extracts are analysed, since other substances, present in unknown concentrations, may affect the assays to a degree which is not always assessable. Gaddum and Lembeck (1949) have shown by statistical calculations that such tests do provide convincing evidence that a mixture of active substances is present but that they do not give a reliable estimate of their concentration.

A method described by Burn, Hutcheon, and Parker (1950), which is a modification of that devised by Bülbring and Burn (1949), appears to be less open to such criticism. In this procedure contractions of a cat's normal nictitating membrane and the rise of blood pressure are recorded. The effects of unknown solutions are compared with those of various equipressor mixtures of the two amines and the percentage methylation calculated from the effect on the nictitating membrane. It is unfortunate that this method is of use only when there is available at least 10 μg. of the combined amines. With this quantity present in 1 ml. of solution, only one estimate of the proportion of adrenaline to *nor*adrenaline can be obtained. For accuracy the mean of several estimates has to be determined.

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Of the chemical methods which have been described, two parallel in principle the pharmacological methods first discussed in that they depend on the difference in the degree of reactivity of the two amines under certain experimental conditions. Both are colorimetric and involve the use of mathematical formulae to translate extinction coefficients into terms of adrenaline and *nor*adrenaline. Euler and Hamberg (1949) measure the intensity of the red colours of adrenochrome and *nor*adrenochrome produced when a mixture of adrenaline and *nor*adrenaline is treated with iodine under carefully defined conditions. Schuler and Heinrich (1948, 1949a) based their method on the yellow colours produced by the catechols when coupled with a relatively complex diazonium salt, again under strictly defined conditions. The employment of these methods is limited, *inter alia*, by their lack of sensitivity, some 20–40 μg. of the combined amines apparently being the minimum required for the determinations.

Goldenberg, Faber, Alston, and Chargaff (1949) have devised a method based on the paper chromatographic separation of *nor*adrenaline and adrenaline, phenol being used as solvent (James, 1948). An estimate of the adrenaline and *nor*adrenaline content is made by planimetry and semi-logarithmic calculation (Fisher, Parsons, and Morrison, 1948) of the Prussian blue spots obtained in the adrenaline and *nor*adrenaline positions in the developed and dried chromatogram by spraying with potassium ferricyanide (James, 1948) followed by ferric sulphate. The limit of sensitivity is not less than 2 μg. for each amine, and, since this quantity must be applied to the paper in a volume of 0.01 ml., it follows that the concentration of the solution must be somewhat high.

From this review of existing techniques for the determination of adrenaline and *nor*adrenaline in mixtures it would appear that, for many pharmacological experiments in which at the most only a few micrograms of the amines are present, none is wholly suitable. The chemical methods lack sensitivity while the biological methods, which in the main are highly sensitive, tend to be deficient in accuracy. This deficiency in accuracy could be overcome to a large extent by first separating the two amines so that each might be assayed individually against a standard solution of the appropriate amine on a suitably sensitive pharmacological preparation.

The account which follows is a description of the adaptation of the paper chromatographic technique described by James (1948) for the qualitative separation of adrenaline and *nor*adrenaline to the quantitative determination of these amines when present together in biological fluids and tissue extracts. The method involves the separation of the two amines by paper chromatography, elution of the separated amines from the appropriate portions of the developed chromatogram, and finally the determination of the adrenaline and *nor*adrenaline contents of the eluates by assay on the rat's blood pressure preparation. The sensitivity of the method appears to be limited only by that of the assay procedure.

METHODS

Separation of adrenaline and noradrenaline *by paper chromatography*

Chromatographic technique.—All chromatograms are carried out by the capillary ascent method of Williams and Kirby (1948), water-saturated phenol being used as solvent. Phenol (500 g.) is distilled at atmospheric pressure from zinc dust (20 g.) and the distillate, while still warm, shaken with a small excess of distilled water in a separating funnel. Sulphur dioxide is bubbled through the mixture for 10 min. and the mixture allowed to stand overnight to permit complete separation of the phases. The lower yellow phenolic layer (500 ml.) is poured into a clean dry glass tank (15 cm. $\times$ 32 cm. $\times$ 45 cm.) to give a layer

of fluid about 1.5 cm. in depth. The tank is closed by a glass plate carrying an inlet and an outlet tube at diagonally opposite corners, so that the air inside the tank can be replaced by another gas. The union of the top of the tank to the glass plate is made airtight by the liberal use of soft paraffin. The solvent may be used repeatedly, but it has been our practice to renew it once a week.

Preparation of the filter paper.—A sheet of Whatman No. 1 filter paper is cut and ruled off in pencil in the manner illustrated in Fig. 1. Two samples, each containing adrenaline and *nor*adrenaline, can be chromatographed on the one sheet of paper. The paper is

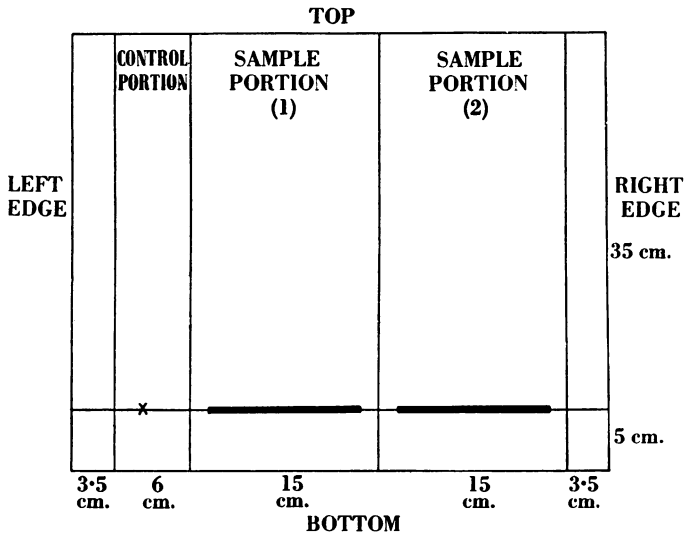

FIG. 1.—Diagram of method of ruling of sheet of filter paper for the chromatographic separation of adrenaline and *nor*adrenaline.

fashioned into cylindrical form by joining the left and right sides edge to edge by cellulose tape (1.6 cm. wide) applied along the length of the join to within about 2 cm. from the bottom of the paper. The edges of the paper at the join must not overlap, as any overlapping results in an uneven solvent flow extending some distance on either side of the join. The sample portions of the paper *only* are damped with a freshly prepared solution of ascorbic acid (50 mg. per 100 ml.). An all-glass spray should be used for this purpose. The paper is allowed to dry at room temperature, about 1.5–2 hr. being required.

Application of the test solution to the ascorbic acid treated paper cylinder.—The material to be analysed—usually in the form of a solution in ethanol acidified with concentrated hydrochloric acid (0.1 ml./100 ml. ethanol)—is applied along the "15 cm." line at the foot of the paper cylinder (thickened line in Fig. 1), care being taken that the fluid does not creep closer than 1.5 cm. to the boundary lines of the " sample portion " of the paper. The solution is best applied from a capillary pipette as a series of drops each approximately 0.005 ml. at a distance apart such that the drops just merge into one another when they spread on the paper. The width of the moisture band should not at any time exceed 1 cm. When the length of the line has been wetted, the alcohol is allowed to evaporate at room temperature and another series of drops applied. This process is continued until all the solution has been transferred to the paper. The whole process takes about an hour.

Aqueous solutions may also be treated in a similar manner, but in this case it is advantageous to apply the solution over the whole width of the two sample portions of the paper. This will balance to some extent the extra time required for the solvent to evaporate. Heat should not be applied to facilitate the evaporation of the solvent.

Finally, on to the " control portion " of the paper at a point 5 cm. from the foot of the cylinder and 2 cm. from the left-hand boundary line (point X in Fig. 1) is applied 0.01 ml. solution of adrenaline and *nor*adrenaline (10 mg. each amine/ml. 0.01 N–HCl).

Development of the chromatogram.—The test and control solutions having been applied, the paper cylinder is stood with its lower end immersed in the solvent in the glass tank. A tank of the dimensions already quoted will accommodate two such paper cylinders quite easily. The tank, standing in a well-ventilated fume cupboard, is closed with the glass plate and sulphur dioxide passed in for 15 min., after which the inlet and outlet tubes are closed. Development of the chromatogram is allowed to proceed for 20–24 hr., when the solvent will have risen some 25–30 cm. up the paper.

Removal of solvent from the developed chromatogram.—The paper cylinder is removed from the tank and the cellulose tape, both backing and adhesive, stripped off. The filter sheet is held at the dry end and pulled backwards and forwards three or four times through a layer of benzene (A.R.) contained in a large porcelain developing dish. Care is taken that the whole of the paper which has been wetted with phenol is washed with the benzene. The paper is then hung up to dry for about half an hour, after which time it may still feel slightly damp but will be sufficiently dry to handle.

Locating adrenaline and nor*adrenaline on the developed and dried chromatogram.*—The " control portion " of the paper is cut off and sprayed with a solution of 0.44 g. $K_3Fe(CN)_6$ in 100 ml. 0.2 M–phosphate buffer *p*H 7.8 (James, 1948). Care must be taken that none of this solution reaches the " sample portions " of the paper. *Nor*adrenaline and adrenaline form red oxidation products and show up on the paper strip as red spots, the *nor*adrenaline spot being the one nearer to the starting line of the chromatogram. The distances from the starting line to the lower and upper extremities of the two spots are measured. These measurements serve to locate the amines on the " sample portions " of the paper. Allowance has to be made, however, for the fact that in the chromatography of biological extracts the flow of the amines may be influenced by other materials present in the extracts. It is advisable, therefore, that a preliminary experiment be carried out in which adrenaline and *nor*adrenaline (0.1 ml. control solution is suitable) is added to an extract and the extract chromatographed in parallel with a normal control. Spraying of both chromatograms with the $K_3Fe(CN)_6$ reagent will give information as to the effect of substances in the extract on the flow of the amines. With plasma and adrenal gland extracts we have found that there is a tendency for the amines to flow slightly further than they do in pure solution. Accordingly, as a routine measure, we have corrected for this difference in the position of the amines in the " sample " and " control portions " of the paper by subtracting 1 cm. from the measurement of the lower extremity and adding 2 cm. to that of the upper extremity of the *nor*adrenaline control spot and subtracting 1 cm. and adding 1 cm. to the lower and upper limits respectively of the adrenaline control spot. Transverse strips conforming to these corrected measurements of the location of the two amines are cut from the " sample portions " of the paper. The strips, usually 6–7 cm. wide, are suitably marked in pencil for future identification.

Elution of the amines from the paper strips.—The amine contained in each strip is eluted therefrom with a solution containing 0.4 g. $NaH_2PO_4.2H_2O$ (A.R.) and 1 mg. ascorbic acid per 100 ml. The method of elution is similar to that described by Dent (1947). Each paper strip is cut to a point at one end, care being taken not to remove any paper on which adrenaline or *nor*adrenaline is likely to be present. The other end is inserted to a depth of about 0.5 cm. between two microscope slides held together by a narrow elastic band. The extruding portion of the strip is bent sharply over the edge of the slides so that it will hang vertically when the far sides of the slides are immersed in the eluting fluid. The pointed end of the paper just intrudes into the mouth of a test-tube or a 15-ml. centrifuge

tube. The eluting fluid passes between the microscope slides, on to and down the paper, the eluate dropping into the collecting tube. To prevent undue evaporation of the solvent from the papers, the elutions are carried out in a fairly airtight chamber containing a beaker of water. The elutions are allowed to continue overnight.

While any suitable vessel may be used as a reservoir for the elution fluid we have found it convenient, when a number of elutions have to be carried out simultaneously, to use a battery of troughs prepared from celluloid tubing. An 8-cm. length of celluloid tubing, 1.5 cm. internal diameter, is sectioned along its length about 1 mm. from a diameter and the deeper section closed at each end by a strip of celluloid. If such a trough be filled with 7.5 ml. eluting fluid, the volume of eluate collected in an overnight run will be 3–4 ml. By thus restricting the supply of eluting fluid, volumes of eluates will be obtained which are fairly constant and the quantity of NaH_2PO_4 in the eluate will give rise eventually to an almost isotonic solution for the biological assay.

Preparation of eluates for biological assay.—To remove traces of phenol each eluate is shaken vigorously three times with equal volumes of benzene (A.R.), the benzene layers after separation being sucked off through a capillary pipette. The aqueous layer is transferred with washing with 1–2 ml. distilled water to a 50 ml. R.B. Quickfit and Quartz flask and evaporated *in vacuo* at 55–60° (external temperature). Heating is stopped when the fluid has evaporated almost but not completely to dryness. The flask and contents are allowed to cool slowly to room temperature with the suction still in operation. The residue in the flask is taken up in 1.00 ml. ascorbic acid solution (50 μg./ml.) and the adrenaline or *nor*adrenaline content of this solution determined by assay on the rat's blood pressure preparation. The solutions are kept below 5° until the assays can be performed.

Assay of adrenaline and noradrenaline *by the rat's blood pressure preparation*

Preparation of the rat.—The rat's blood pressure preparation has been modified from that of Landgrebe, Macaulay, and Waring (1946).

A rat (200–250 g.) of either sex is anaesthetized with urethane (175 mg. or 0.7 ml. of a 25 g./100 ml. aqueous solution per 100 g. body weight subcutaneously) and then tied in a supine position to a board. Through a mid-line incision in the neck the trachea is cannulated and one carotid artery dissected ready for cannulation. The other carotid artery is tied off and loose ligatures placed round both vagi. A cannula is tied into a femoral vein and fixed firmly by a ligature embracing it and the thigh together. The distal end of the venous cannula is joined by a short piece of rubber tubing to a 1-ml. burette fitted, just above the tap, with a side arm which is connected by means of a piece of rubber tubing with a spring clip to a reservoir containing warm saline. The burette can thus be refilled without detaching it. Heparin (2 mg./100 g. rat) is injected through the venous cannula and washed in with saline. The arterial cannula is then tied in, fixed in a clip, and connected to a special mercury manometer, normal saline being used as the liquid bridge. Both vagi and the femoral nerve lateral to the venous cannula are cut. Before commencing the assays the preparation is left for 15–20 min. until the blood pressure settles to a steady level. Once set up the rat should not be touched, especially when injections are made. The slightest handling causes marked changes in the blood pressure of the animal.

All the cannulae used in this preparation are of the conventional pattern but of suitable size, with orifices of about 2.5 mm. external diameter for the tracheal cannula and 1 mm. external diameter for the arterial and venous cannulae, the internal diameters being slightly less. The special mercury manometer used is the subject of an addendum to this paper.

Preparation of standard solutions of adrenaline and noradrenaline.—Standard solutions of adrenaline (2 μg./ml.) and *nor*adrenaline (1 μg./ml.) are made up from stock solutions by dilution in normal saline containing freshly dissolved ascorbic acid (50 μg./ml.). The

stock solutions of the amines, each containing 1 mg./ml. of the base, were prepared from synthetic (−) adrenaline (B.W. and Co.) and (−) *nor*adrenaline (+) bitartrate monohydrate (Bayer) by solution in 0.01 N–HCl. Such stock solutions appear to remain stable for several months if stored in small stoppered test-tubes at 5°.

Assay routine.—Test samples containing adrenaline are assayed against standard adrenaline and those containing *nor*adrenaline against standard *nor*adrenaline solutions. If required, dilution of a test solution is made in normal saline containing freshly dissolved ascorbic acid (50 μg./ml.). Test and standard solutions, both at room temperature, are injected alternately into the venous cannula of the rat preparation by means of a 1-ml. tuberculin syringe. The volume injected may be varied from 0.025 ml. to 0.4 ml., the dose being immediately washed in with a quantity of normal saline such that the total volume of fluid given at any one time is 0.6 ml. This total volume of fluid may be varied with the circumstances but should, of course, be the same for both test and standard in any one assay. In our hands the greatest accuracy is obtained when the total volume does not exceed 0.4 ml. Injections are made at regular intervals, usually every two minutes. It has been our custom in carrying out these assays to keep the dose of the test solution constant, and to bracket and finally match the responses with varying doses of the standard solution. An illustration of a record from a typical assay by the rat's blood pressure preparation is given in Fig. 2.

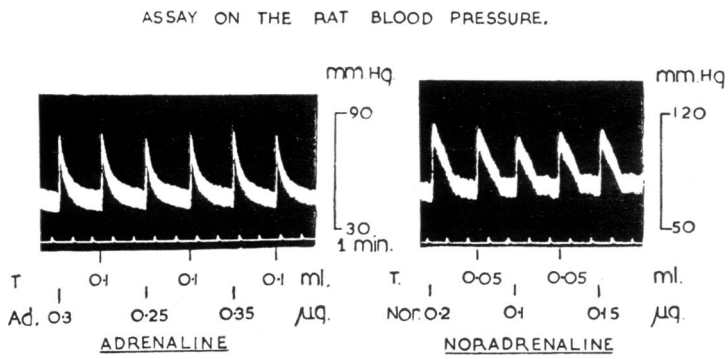

FIG. 2.—Illustrations of typical records obtained in the assay of adrenaline and *nor*adrenaline by the rat's blood pressure preparation.

RESULTS

Application of the method to the quantitative separation of adrenaline and nor*adrenaline in simple solution, biological fluids, and tissue extracts*

The results quoted below are representative of those obtained by the application of the method to the analysis of simple solutions and extracts containing adrenaline and *nor*adrenaline. The figures given in parentheses in the Tables are the upper and lower limits of the estimates of the assay, the smaller figure being definitely less than the true value of the unknown and the larger definitely greater as observed from the blood pressure responses.

Simple solution.—Mixtures of adrenaline and *nor*adrenaline in 1.00 ml. acid-ethanol (0.1 ml. conc. hydrochloric acid/100 ml. ethanol) were analysed. The recoveries are shown in Table I.

Plasma.—Blood was obtained from rabbits, after the injection of 5 mg. heparin, by bleeding from an ear vein into a beaker surrounded by ice. The plasma was

TABLE I

RECOVERY OF ADRENALINE AND *nor*ADRENALINE FROM MIXTURES IN SIMPLE SOLUTION

Mixture	Adrenaline			*Nor*adrenaline		
	Added μg.	Found μg.	Recovery %	Added μg.	Found μg.	Recovery %
	Series 1. Recovery experiments carried out at different times					
a	1.0	0.75 (0.5 – 1.0)	75	10.0	7.5 (5.0–10.0)	75
b	1.0	1.6 (1.33– 2.0)†	160	10.0	7.5 (5.0–10.0)	75
c	2.0	2.0 (1.33– 4.0)	100	Nil	<0.062*	—
d	2.0	2.0 (1.33– 4.0)	100	Nil	<0.062*	—
e	2.0	2.0 (1.5– 2.5)	100	Nil	<0.062*	—
f	2.0	1.5 (1.0–2.0)	75	Nil	<0.062*	—
g	10.0	8.0 (6.0–12.0)	80	1.0	0.75 (0.5–1.0)	75
h	10.0	15.0 (10.0–20.0)†	150	1.0	0.75 (0.5–1.0)	75
Series 2.	Recovery experiments done simultaneously. All assays carried out on the same rat preparation					
j	0.25	0.19 (0.13–0.25)	75	0.13	<0.25*	—
k	0.50	0.38 (0.25–0.5)	75	0.25	<0.25*	<100
l	1.0	1.0 (0.75–1.5)	100	0.50	0.31 (0.25–0.38)	62
m	2.0	2.0 (1.0–4.0)	100	1.0	0.75 (0.5–1.0)	75
n	2.0	2.0 (1.5–2.5)	100	0.10	<0.25*	—
p	2.0	2.0 (1.5–2.5)	100	0.25	<0.25*	100
q	2.0	2.5 (2.0–3.0)	125	0.50	0.25 (0.13–0.38)	50
r	20.0	15.0 (10.0–20.0)	75	1.0	0.75 (0.5–1.0)	75
s	Nil	<0.25*	—	Nil	<0.25*	—

* This figure represents the minimum quantity of amine/ml. solution which on injection of 0.4 ml. into the rat preparation would give a pressor response just noticeably different from that produced by the injection of an equal volume of saline. Thus concentrations below this figure cannot be assayed.

† Discrimination of rat preparation poor.

separated by centrifugation (3,000 r.p.m. for 10 min.) in a tube packed in ice. Adrenaline-*nor*adrenaline mixtures in plasma were made up by diluting 0.25 ml. of an appropriate standard solution of adrenaline and a like volume of *nor*adrenaline solution in 0.01 N–HCl with 2 ml. plasma. Of this mixture, 1 ml. was taken for analysis.

For the paper chromatography it was necessary that the protein be first removed from the plasma. This was effected by precipitation with acid-ethanol. To 10 ml. cold acid-ethanol (0.05 ml. conc. HCl/100 ml. ethanol) in a 15-ml. centrifuge tube, 1 ml. adrenaline-*nor*adrenaline-plasma mixture was added drop by drop. The tube was stoppered and the mixture thoroughly shaken and left at 5° for 1.5 hr. The underside of the stopper was washed into the main bulk of the fluid with 1 ml. acid-ethanol and the mixture centrifuged 15 min. at 4,000 r.p.m. The clear and colourless supernatant was transferred to a 50-ml. Quickfit and Quartz R.B. flask, the inside walls of the tube being washed down into the flask with 2 ml. acid-ethanol. The solution was evaporated to dryness *in vacuo* at 55–60° (external temperature), the flask being allowed to cool slowly to room temperature before releasing the suction. The faintly yellow lipid-like residue was leached with 0.75 ml. acid-ethanol (0.1 ml. conc. HCl/100 ml. ethanol) and the extract applied to a filter-paper cylinder. A further extract with 0.5 ml. acid-ethanol was also applied. Results obtained with this procedure are given in Table II.

TABLE II

RECOVERY OF ADRENALINE AND *nor*ADRENALINE FROM MIXTURE IN PLASMA

The results for individual members of each group of experiments were obtained from assays on the same rat preparation. 1 ml. plasma-adrenaline-*nor*adrenaline mixture was used for each analysis

Sample	Adrenaline			*Nor*adrenaline		
	Added μg.	Found μg.	Recovery %	Added μg.	Found μg.	Recovery %
1a	10.0	10.0 (6.7–12.3)	100	2.0	1.5 (1.0–2.0)	75
1b	2.0	2.0 (1.5–3.0)	100	10.0	7.5 (5.0–10.0)	75
1c	2.0	2.0 (1.5–3.0)	100	10.0	7.5 (5.0–10.0)	75
1d	Nil	<0.5*	—	Nil	<0.13*	—
2a	10.0	10.0 (7.5–15.0)	100	2.0	1.5 (1.0–2.0)	75
2b	10.0	10.0 (7.5–15.0)	100	2.0	1.5 (1.0–2.0)	75
2c	2.0	2.0 (1.5–3.0)	100	10.0	5.0 (3.3–6.7)	50
2d	2.0	2.0 (1.5–3.0)	100	10.0	7.7 (6.7–10.0)	77
2e	Nil	<0.25*	—	Nil	<0.13*	—
3a	10.0	10.0 (7.7–12.5)	100	1.0	1.0 (0.5–1.5)	100
3b	1.0	0.8 (0.67–1.0)	80	10.0	10.0 (5.0–15.0)	100
3c	Nil	<0.25*	—	Nil	<0.13*	—

* See footnote to Table I.

Adrenal glands.—As an example of the application of the method to tissue extracts, some results obtained in the analyses of adrenal glands from 15 normal rats are given in Table III. The standard deviations shown in this test are an indication of the variability of the rats and not of the error of the method.

TABLE III

DETERMINATION OF ADRENALINE AND *nor*ADRENALINE IN ADRENAL GLANDS OF NORMAL RATS

Mean estimates ± standard deviation from 15 rats (150–200 g.)

Amine	μg./100 g. body weight	μg./mg. gland	Percentage combined amines (mean value)
Adrenaline	20.97±4.41	0.965±0.291	93
*Nor*adrenaline ..	1.63±0.50	0.077±0.035	7

The weight of an adrenal gland was 22.9±5.9 mg./100 g. rat.

Estimates from 10 newborn rats (<24 hours) in groups of 5 each.

Adrenaline .. .. 0.225 : 0.375 μg./gland

*Nor*adrenaline .. .. 0.031 : 0.025 „ „

The rats, weighing 150–200 g., were starved overnight and killed by a blow on the head. Both adrenals from each rat were removed quickly, weighed, and ground with 0.5 ml. 0.15 N–HCl. Ethanol (10 ml.) was added with thorough mixing. After standing 30 min. at room temperature the mixture was centrifuged for 15 min. at 2,000 r.p.m. The clear supernatant was decanted into a 50-ml. Quickfit and Quartz R.B. flask, the residue being washed with 2.5 ml. ethanol. The alcoholic extract was evaporated to dryness *in vacuo* at 55–60° (external temperature). The reddish-brown

residue was leached with 0.75 ml. acid-ethanol (0.1 ml. conc. HCl/100 ml. ethanol) and the extract applied to a paper cylinder. A further extract with 0.5 ml. acid-ethanol was also applied. Each analysis was thus carried out on the pooled glands of a single rat.

The adrenals of newborn rats (less than 24 hr. old) were also analysed, all the glands of five animals being extracted together in 0.5 ml. 0.15 N–HCl.

From the results of these analyses of normal rat's adrenals it is of interest to note that, of the various species of animal for which figures are available, namely the cat, guinea-pig, rabbit, and ox (Schuler and Heinrich, 1949b; Euler, Hamberg, and Purkhold, 1949), the rat has the lowest average percentage of *nor*adrenaline in the pressor amines of the adrenal medulla.

Analysis of a phaeochromocytoma.—Through the kindness of Professor Greenfield and his colleagues of the Royal Victoria Hospital, Belfast, we have had an opportunity of analysing an extract of a phaeochromocytoma by our method. A portion of the tumour was received in 0.1 N–HCl. It was cut into small pieces and reduced to a pulp by grinding in a glass mortar with some of the acid. The addition of a little acid-washed silver sand aided the disintegration. After thorough mixing of the pulp and the acid the mixture was centrifuged at 2,000 r.p.m. for 10 min. The faintly opalescent brown supernatant, *p*H 4 (glass electrode), was stored at 5° overnight. For the determination of the adrenaline and *nor*adrenaline content of the extract some of the fluid was centrifuged at 3,000 r.p.m. for 15 min. to give a clear brown supernatant over a small amount of dark-brown precipitate. Measured quantities (0.05 ml. and 0.10 ml.) of this supernatant were applied to a filter-paper cylinder by means of a graduated 0.2-ml. pipette fitted with a capillary tip, and put through the analytical procedure.

TABLE IV

DETERMINATION OF ADRENALINE AND *nor*ADRENALINE OF A HUMAN ADRENAL MEDULLARY TUMOUR

(5.26 ml. extract ≡ 1 g. tumour)

Vol. of extract analysed ml.	Quantity of amine found	
	Adrenaline μg.	*Nor*adrenaline μg.
0.05	10.0	67
0.10	22.5	133

By calculation from the mean of these assays the tumour contained 7.0 mg. *nor*adrenaline and 1.1 mg. adrenaline/g.

The results for these assays are given in Table IV. It will be seen that the results for *nor*adrenaline in the two samples are in complete agreement while those for adrenaline do not differ by more than 12.5 per cent, which testifies to the accuracy of the method even when relatively large quantities of the amines are present.

In passing it may be mentioned that the proportion of adrenaline to *nor*adrenaline found in this tumour was similar to those recorded in the literature (Holton, 1949; Goldenberg *et al.*, 1949).

Discussion

Adrenaline and *nor*adrenaline in mixtures in simple solution, biological fluids, and tissue extracts can be determined with a high degree of accuracy by the method described above. This method involves the separation of the amines by paper chromatography, elution of the separated amines from the developed chromatogram, and the assay of the separated amines in the eluates by the rat's blood pressure technique. In our hands the procedure has given consistently better and more dependable results than the method of parallel quantitative assays on the isolated uterus and colon of the rat described by Gaddum and Lembeck (1949). In control experiments recoveries of 75–125 per cent have been obtained from pure solutions and from plasma with quantities of adrenaline and *nor*adrenaline ranging from 0.25 μg. to 20 μg. and with either amine in twentyfold excess. It is our belief that the sensitivity, and also to a great extent the accuracy, of the method is limited only by the final assay procedure.

In the course of the development of the method the isolated uterus and colon of the rat and the perfused rabbit's ear (Gaddum, Peart, and Vogt, 1949) had been used for assay purposes, but these were eventually abandoned since they appeared liable to yield erroneous results owing to interference from traces of reagents used during the separation of the amines. The rat's blood pressure was found not to be subject to such interference. Accurate assays of solutions of *nor*adrenaline containing 0.125 μg./ml. and adrenaline solutions containing 0.25 μg./ml. can be made with a sensitive preparation. The ratio of the dose of (−) *nor*adrenaline to the equivalent dose of (−) adrenaline averages 0.25 (0.15–0.75). Rats of either sex weighing 200–250 g. may be used. Smaller animals, though more sensitive, are irregular in their responses, while larger animals are relatively insensitive.

The rat's blood pressure preparation appears to be one of the most sensitive tests for *nor*adrenaline, as little as 0.062 μg. often being determinable. It is thus comparable in sensitivity to the isolated colon of the rat, hitherto regarded as the best biological test for *nor*adrenaline; it has, in addition, the advantage that it is more accurate than the colon preparation. Statistical treatment of assay results by the method of Bliss (1944) and Noel (1945) gave a mean value for λ of 0.096 compared with 0.126 found by Gaddum and Lembeck (1949) for the colon preparation.

It is considered that by the inclusion of the paper chromatographic separation the analytical procedure as a whole gains in objectiveness, since the amines behave like adrenaline and *nor*adrenaline not only pharmacologically but also physicochemically in that they are located on the paper chromatogram at or close to the positions occupied by pure adrenaline and *nor*adrenaline. Further, the chromatography will effect a considerable purification of the extract containing the amines so that the assays will be less liable to interference from other substances in the biological material than when more direct methods of assay are employed.

When the idea of adapting the paper chromatographic separation technique of James (1948) to the quantitative separation of adrenaline and *nor*adrenaline was first considered it was thought that the most likely source of loss would be through oxidation of the amines during the relatively protracted development of the chromatogram. It was with this in mind that the spraying of the paper with ascorbic

acid and the development of the chromatogram in an atmosphere of sulphur dioxide were introduced at the outset. We have not investigated whether either or both these precautions are necessary.

Experiments in which the phenol was removed from the developed chromatograms by heating them at 40° in an oven, through which a stream of nitrogen was passed to sweep out the vapour, showed that such a procedure led to variable results. The detrimental effect of heat on the quantitative recovery of amino-acids from paper chromatograms has also been reported (Fowden and Penney, 1950).

For the application of the method to plasma it is necessary, as a first step, to remove the protein. Some considerable difficulty was experienced in finding a suitable method. It has been reported in the literature that precipitation with trichloroacetic acid or metaphosphoric acid is unsuitable since adrenaline, and presumably *nor*adrenaline, is partly or completely removed from the solution on the protein precipitate (Gaddum and Schild, 1934; Jörgensen, 1945; Lund, 1949). A method of deproteinization used by Folin, Cannon, and Denis (1912) and Frowein (1923) for the extraction of the pressor amines from adrenal glands proved unsatisfactory when applied to plasma. This method entails the denaturation of the protein with acid at 100°, cooling, adjustment of the solution to the isoelectric point of the denatured protein by the addition of sodium acetate, and finally coagulation by heating. The recoveries were variable, and interference was experienced in the assay of the *nor*adrenaline fractions owing probably to the presence of acetate (Bauer and Richards, 1928). Replacement of the acetate by bicarbonate, citrate, or phosphate did not materially improve the recoveries. Also examined but found unsuitable was a modification of a method recently described by Lund (1949) in which adrenaline and *nor*adrenaline are removed from plasma by adsorption on chromatographic alumina (B.D.H.) and eluted therefrom with acid. Of the various methods investigated only the acid-ethanol method which has been described gave consistently good recoveries.

Summary

1. A method is described for the quantitative separation of adrenaline and *nor*adrenaline in pure solution, biological fluids, and tissue extracts.

2. The method depends on the separation of the amines by paper chromatography, elution of the separated amines from the developed chromatogram, and the assay of the separated amines in the eluates by means of the rat's blood pressure preparation.

3. Recovery experiments from pure solution and from plasma showed that the amines can be estimated in quantities as low as 0.25 μg. with an accuracy of 75–100 per cent, this level of accuracy still holding when one of the amines is in twenty-fold excess in a mixture.

4. It is considered that the combination of sensitivity, accuracy, and objectiveness which the procedure possesses makes it capable of a breadth of application not to be found in the hitherto published methods for the determination of adrenaline and *nor*adrenaline in mixtures.

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ADDENDUM

A MODIFICATION OF THE CONVENTIONAL MERCURY MANOMETER FOR BLOOD-PRESSURE RECORDINGS

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The mercury manometer used in these experiments is described here because it has been found that the details of the apparatus are especially important in experiments on rats.

The glass portion of the manometer is constructed of thick-walled tubing with an internal diameter of 2–3 mm. The total height of one limb is 24 cm.

The writing point is made of a piece of glass capillary about 9 cm. long and bent in the middle at right angles. The point of one limb writes on the smoked paper and the other limb is in contact with a vertical piece of glass capillary suspended at least 20 cm. above the top of the manometer and adjusted so as to keep the writing point in contact with the paper.

This writing point is attached to the upper end of a length of stainless steel hypodermic needle tubing (about 20 cm. of No. 23 S.W.G.; approximately 0.6 mm. in diameter) passing through a float made of ebonite or polythene tubing. This float fits the glass tubing as closely as possible without sticking, and is mounted about 4 cm. from the lower end of the steel tubing, which is plugged with a piece of wire. The float rests on the surface of the mercury and the lower part of the steel tubing is submerged. This submerged portion is kept central by the float and the usual brass cap on the top of the glass tube through which the upper part of the steel tubing passes. The length of the submerged

part is so adjusted that the upward thrust of the mercury permits the float to rest lightly on the surface of the mercury and to maintain it in that position during all movements of the mercury column. Aluminium wire may be substituted for the steel tubing, in which case the submerged portion of the wire should be well lacquered.

Polythene tubing 3.50 mm. bore is used to connect the B.P. cannula to the manometer. This has several advantages, the main one being that there is practically no loss in the transmission of the impulse from the cannula to the manometer. This is certainly not true when using rubber tubing unless of very heavy wall.

This type of manometer has been found to give very satisfactory records in rat's blood pressure experiments. It is of simple and robust construction, the use of relatively heavy materials for the float and attachments being permitted by the nature of the design.