THE ESTIMATION OF NORADRENALINE IN URINE AND ITS EXCRETION IN NORMAL AND HYPERTENSIVE SUBJECTS

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Recognition of the association of paroxysmal hypertension with phaeochromocytomata, actively secreting tumours of the chromaffin tissue of the adrenal medulla, has resulted in the need for more careful scrutiny of hypertensive patients for evidence of this disease which, if found, may be cured by surgical removal of the growth.

Much help in diagnosis may be obtained by the trial of provocative agents such as histamine, or of blocking, adrenolytic drugs like "rogitine," but false results are not uncommon and the diagnosis may be left in doubt.

Following the demonstration by Holton (1949) that noradrenaline is present in the adrenal medullary tumours, Engel and von Euler (1950) reported that the excretion of the catecholamines adrenaline and noradrenaline in the urine is markedly increased in the presence of a phaeochromocytoma and this is now the most reliable diagnostic method. Unfortunately, the complexity of the methods for determining these substances in the urine has put them beyond the capacity of most clinical laboratories, and it was with the object of evolving a relatively simple clinical method that this work was undertaken.

Two methods for the determination of catecholamines are available: bio-assay, which is satisfactory but involves the use of an animal preparation as a test-object for the pressor activity of urine extracts, and the fluorimetric, for which a fluorimeter of sufficient sensitivity has not, until recently, been commercially available in this country.

Fluorimetric methods for the determination of catecholamines in blood have been described by Lund (1949), Weil-Malherbe and Bone (1952, 1953, 1954) and in urine by Goldenberg, Serlin, Edwards, and Rapport (1954) and von Euler and Floding (1955a, 1955b). Weil-Malherbe and Bone constructed their own fluorimeter, and a similar

apparatus was used by von Euler and Floding; Lund employed a Coleman 12B fluorimeter, which in a modified form was also used by Goldenberg *et al.*

Method

Separate determination of adrenaline and noradrenaline increases the time and labour involved, and since it is not essential for diagnostic purposes it will not be described in this paper; methods are described by Lund (1950), Goldenberg *et al.* (1954), and von Euler and Floding (1955a).

The principle of the method is as follows. The acidified urine is heated to hydrolyse the conjugated catecholamines, which are then adsorbed on aluminium hydroxide and eluted with a mixture of acetone and ethanol (von Euler and Hellner, 1951; Goldenberg *et al.*, 1954). A sample of the eluate, after concentration, is subjected to paper chromatography with *n*-butanol-acetic acid-water (Shepherd and West, 1953) and the separated catecholamines eluted with dilute HCl. Fluorimetric analysis is performed at pH 6 by the method of von Euler and Floding (1955a).

We have measured the fluorescence with a Hilger Spekker fluorimeter H 764, which is fitted with a 125-watt mercury vapour lamp and a selected Mazda type 27 M 3 visible photo-multiplier. A Cambridge spot galvanometer was used with a low-powered lens in front of the scale to increase the precision of adjustment to the null-point.

With adrenaline and noradrenaline greatest sensitivity is obtained by the use of Chance OB 10 glass (440 m μ) as a primary filter, but we found, in agreement with Lund (1949), that with this filter the blank values of the urine extracts are unduly large. We have, therefore, used Hilger 556 filters (Wood's glass, 365 m μ) throughout this work. On the left-hand, compensating side of the instrument one neutral filter (Hilger 508) was also necessary. Chance OY 4 glass, which cuts off light below 510 m μ , served as a secondary filter. Calorex heat-resisting filters were placed on each side of the light source. All filters were 2 mm thick. The "coarse" sensitivity of the power unit had to be set at 9.

Calibration Graph for Noradrenaline.—Standard solutions were prepared from L-noradrenaline bitartrate and estimated by the method of von Euler and Flooding (1955a). Oxidation with the ferricyanide was allowed to proceed for three minutes, and 15 minutes after addition of the sodium hydroxide reagent readings were made against a solution of quinine sulphate containing 0.075 μg . per ml. The transmission scale of the fluorimeter was used (%tr.).

The quinine sulphate was made by diluting 0.75 ml. of a stock solution containing 20 mg. quinine sulphate dissolved in 1,000 ml. N/10 sulphuric acid to 200 ml. with N/10 sulphuric acid. These solutions are stable.

The reagent-blank values ranged from 10 to 12% tr. Sodium hydroxide without ascorbic acid was used in the blanks for these standard values as for the assay of urine extracts. If the ascorbic acid-sodium hydroxide reagent is used the values obtained are about 2% tr. higher; it is for this reason that the graph does not pass through the origin (Fig. 1).

Adrenaline.—Under the same conditions we have found that, weight for weight, % tr. values for

adrenaline at pH 6 are approximately 30% higher than those given by noradrenaline.

Extraction of Catecholamines from Urine.—The patients should not be given medicines or vitamins during the period of urine collection, since these might give high blank values on estimation.

Twenty-four-hour specimens of urine are collected in bottles containing 10 ml. concentrated hydrochloric acid and kept in a cool place. The volume of the specimen is noted, and if less than 1,500 ml. it is made up to this figure with water.

The urine is filtered and 500 ml. measured into a litre flask. The reaction is adjusted to pH 1.5–2.0 with concentrated hydrochloric acid, using short range test-paper and the flask immersed in boiling water for 30 minutes. The flask is shaken occasionally and the reaction readjusted, if necessary, with hydrochloric acid. The contents of the flask are then cooled under running water.

The urine is transferred to a litre beaker and 8 ml. of 20% aluminium sulphate (A.R.) is added. While the fluid is vigorously agitated by a magnetic stirrer 3N sodium hydroxide is added drop-wise at a rate of about 60 drops per minute until the pH, continuously determined with a glass electrode, reaches 7.7. In most cases a precipitate of aluminium hydroxide begins to form at about pH 4 and becomes dense as the end-point is reached. With some specimens of urine, however, little or no precipitate forms even at pH 6–7, and it is then necessary to add a further 8 ml. of aluminium sulphate and continue the addition of sodium hydroxide to pH 7.7. Rarely, a third addition of aluminium sulphate may be necessary to produce a satisfactory precipitate as evidenced by dense cloudiness of the liquid.

After stirring for one minute at pH 7.7 the precipitate is immediately collected by centrifuging at 1,800 r.p.m. for 10 minutes in two 250 ml. bottles. A deposit having a total volume of 10–20 ml. is satisfactory. Without delay the precipitate is dissolved by adding 1 ml. of 6N sulphuric acid to each bottle, closing with a stopper and shaking vigorously for a few seconds. If the mixture does not become clear a few more drops of sulphuric acid are added. The brown solution is transferred quantitatively with a small quantity of water to a 100 ml. conical flask and the reaction brought to pH 3.0 (glass electrode) by the addition of 3N sodium hydroxide. Great care is necessary during this procedure to avoid local high alkali concentration. The alkali is added drop-wise from a fine pipette during brisk agitation of the flask, especially after each drop of alkali.

FIG. 1.—Each point is the mean of six determinations. The coefficients of variation (%) of the fluorimeter readings (tr.%) in ascending order from 0.025 μg . were: 4.3, 4.2, 4.7, 2.2, 4.3, 5.3, and 2.6.

When the pH reaches about 3.0 and re-precipitation of the aluminium hydroxide has begun, neutralization is continued with N sodium hydroxide to pH 3.7.

The volume of the creamy mixture is roughly measured and it is transferred with four volumes of ethanol acetone (1:1) to a 250 ml. flask, shaken, and placed in a refrigerator (4° C.) overnight.

Paper Chromatography.—Two 1 ml. samples of the eluate are subjected to paper chromatography. The ethanol-acetone mixture is shaken and its volume measured; the volume occupied by the precipitate is ignored. A portion is centrifuged and 1 ml. of the clear supernatant fluid is measured into a 15 ml. conical centrifuge tube. One drop of 5% acetic acid is added. The tube is placed in a rack in a sloping, almost horizontal position and the fluid concentrated by directing a stream of compressed nitrogen on to the surface from a capillary tube at room temperature. The acetone and ethanol are quickly removed, and after about 10 minutes there remains a small residue of about 0.3 ml.

In the subsequent chromatography a marker is needed to locate the position of the noradrenaline. A further quantity of 1 ml. of the eluate in another tube, to which is added about 5 µg. of noradrenaline (0.05 ml. of 0.01% noradrenaline in 5% acetic acid), is concentrated as described above. If a number of different eluates is being run on one paper a sample of any one will serve as a marker.

The catecholamines are separated by ascending, one-dimensional, chromatography. We have found Whatman No. 41 paper (for chromatography), which is HCl- and HF-washed, to give the best results. We use 12 in. by 12 in. sheets which, after folding concertina-wise, are cut to form seven connected strips or lanes some 24 cm. long and 3 cm. wide. The size of the paper and the number of lanes will depend on the number of specimens and the apparatus available; a movement of the solvent front of about 25 cm. is desirable.

A pencil line is drawn parallel about 3.5 cm. from one edge to traverse the lanes at about 1 cm. from their origins. Using a capillary pipette and teat the concentrated eluates are applied along the pencil lines by repeated applications, drying with a fan at room temperature. The fluid should not be allowed to spread more than about 0.5 cm. on each side of the line. Finally, the tubes are rinsed twice with two drops of water and the washings transferred to the paper.

The paper is suspended with the lower end in the solvent prepared as described by Goldenberg *et al.* (1954): 4 parts of *n*-butanol, 1 part of glacial acetic acid (A.R.), and 5 parts of distilled water are shaken vigorously in a separating funnel; after separation the upper layer is transferred to a centrifuge tube and placed in the refrigerator for about 30 minutes; the mixture is then centrifuged and the clear fluid poured

into the trough of the tank. The lower, aqueous, phase is transferred to a beaker and placed in the tank. The paper is developed at room temperature until the solvent reaches the top, conveniently overnight.

The operations described should be carried out in diffused light and the chromatography tank covered with a cloth or kept in the dark.

Elution of Catecholamines.—The sheet is removed from the tank and dried for one hour in a current of air at room temperature and in diffused light. The lane containing the marker is sprayed with solution containing 0.44% potassium ferricyanide in 0.2 M phosphate buffer pH 8.3, adjacent lanes being carefully shielded. The position of the noradrenaline will be indicated by a pink band some 6 cm. from the line of application.

Using a ruler parallel pencil lines are drawn across the paper lanes about 1.5 cm. on each side of the noradrenaline band. The paper enclosed between the pencil lines is cut out, folded concertina-wise some seven times and dropped into 15 ml. stoppered tubes containing 10 ml. N/1,000 hydrochloric acid. The tubes are placed in a shaker in a horizontal position, protected from light, and gently rocked about 60 times a minute for one hour.

Estimation of Catecholamines.—After elution and without delay two 4 ml. samples, one of which serves as a blank, are delivered into cylinders graduated at 10 ml. and containing 1 ml. of acetate buffer pH 6.0. After making the volume up to 8 ml. with water the estimation is carried out at pH 6 as described by von Euler and Floding (1955a).

If the readings are within the range of the standard graph the duplicate eluate is assayed in a similar manner; if the amount of catecholamines is too high a smaller amount of eluate (2 or 1 ml.) is taken for estimation.

Calculation:

$$\text{noradrenaline} \times \frac{\text{vol. eluate}}{\text{vol. applied to paper}} \times \frac{10}{\text{vol. eluate of paper taken}} = \mu\text{g. per 500 ml. urine.}$$

The mean value of the duplicate estimations is used for calculation.

Accuracy of Method.—In order to test the accuracy of the method 11 duplicate 500 ml. samples of urine were assayed. The total catecholamines present, expressed as noradrenaline, ranged from 15 µg. to 40 µg., mean 26 µg.; the S.D. for a single determination was ± 1.9 µg.

Recovery for Noradrenaline from Urine.—Duplicate urine samples of 500 ml., to one of which noradrenaline was added in amounts of 10 to 1,000 µg., were assayed. The results, shown in Table I, show that recoveries ranged from 79% to 109%, with a mean value of 92%.

TABLE I
RECOVERY OF NORADRENALINE FROM
500 ML. URINE

Noradrenaline Added (μ g.)	Recovery (%)
10	100
10	109
20	85
50	95
50	90
50	93
100	102
100	80
100	80
150	98
150	94
150	100
250	99
250	81
250	80
500	96
500	85
500	79
1,000	100
Mean 92	
S.D. ± 9	

Comparison of Biological and Fluorimetric Methods

The pressor activity of a number of samples of urine was determined on the cat preparation. The animals were anaesthetized with chloralose and 2 mg. per kg. of atropine was injected intravenously followed by 0.1 mg. per kg. of ergotoxine intramuscularly.

Ethanol-acetone eluates, which had been fluorimetrically assayed, were evaporated *in vacuo* as described by Goldenberg *et al.* (1954) and the residues taken up in 0.01 N hydrochloric acid. After suitable dilution they were injected into the femoral vein of the cat preparation and the effects compared with those produced by known amounts of L-noradrenaline. The results are given in Table II. The difference between the two series of results is not significant ($P=0.7$).

TABLE II
COMPARISON OF BIO-ASSAY AND FLUORIMETRY (μ G.
NORADRENALINE PER 500 ML. URINE)

Specimen No.	Bio-assay	Fluorimetry
1	25	31
2	40	45
3	20	18
4	18	31
5	20	25
6	15	29
7	22	24
8	30	30
9	20	22
10	20	24
11	15	24
12	20	21
13	40	60
14	190	200
Mean	35	42

The figures are no more than an approximation because the adrenaline present would, as compared with noradrenaline, exercise a smaller pressor effect on the cat and yield a higher fluorimetric reading. Since, however, the adrenaline content of the urine of the patients represented in Table II would be expected to be small the results are probably not appreciably distorted.

The Catecholamine Excretion of Normal and Hypertensive Subjects

The urinary output of total catechols as noradrenaline was determined in normal subjects and hypertensives (Table III). The mean value for the normals was 89 μ g. per 24 hours (S.D. ± 26) and for hypertensives 90 μ g. per 24 hours (S.D. ± 36); there is clearly no difference between these groups.

Holtz, Credner, and Kroneberg (1947) obtained values of 100 to 150 μ g. of adrenaline or noradrenaline per day and stated that some subjects with essential hypertension excreted larger amounts. In a series examined by Burn (1953) bio-assay gave 15–150 μ g. for normotensive adults with a mean value of 75 μ g.; hypertensives did not give higher figures. Von Euler and Hellner (1951), also by bio-assay, found the total catecholamine excretion in healthy students to be 19–69 μ g. per day. In a group of hypertensives von Euler (1952) found 66% to excrete less than 58 μ g. noradrenaline in 24 hours and 32% up to 200 μ g., while Goldenberg *et al.* (1954) report values obtained by bio-assay of 11 to 50 μ g. per day for normals and up to 100 μ g. for essential hypertensives. These results, suggesting that hyper-

TABLE III
TOTAL CATECHOLAMINE EXCRETION AS NORADREN-
ALINE (μ G./24 HOURS) IN NORMAL AND HYPERTENSIVE
SUBJECTS

Normal				Hypertensive			
Sex	Age	Blood Pressure (mm. Hg)	Catecholamines	Sex	Age	Blood Pressure (mm. Hg)	Catecholamines
F	22	110/70	135	M	81	150/80	119
F	27	110/75	33	F	61	165/95	54
F	48	120/75	100	F	54	170/90	121
M	59	120/80	94	F	44	170/110	77
M	50	130/75	92	F	71	170/100	45
M	53	130/75	94	F	45	170/110	63
M	55	130/80	105	M	33	175/100	90
M	52	135/80	92	F	65	175/100	63
M	55	135/80	57	M	64	180/115	135
F	30	135/80	87	M	45	180/110	69
M	44	135/90	93	M	56	180/110	130
M	52	135/80	85	M	58	190/110	135
				M	49	240/120	149
				M	54	250/120	87
				M	43	260/120	60
				F	68	300/155	45
Mean	45		89		56		90
			S.D. ± 26				S.D. ± 36

tensive subjects excrete larger amounts of catecholamines than normals, are not confirmed by the results of Burn (1953) and those recorded in Table III.

We have had an opportunity to examine one case only of phaeochromocytoma while this work was in progress. Fluorimetric estimation of the total catecholamines as noradrenaline showed an excretion of 2,600 $\mu\text{g.}$ in 24 hours.

Discussion

Extraction of catecholamines from urine by adsorption on aluminium hydroxide, as first described by Shaw (1938) and modified by von Euler (1948), is still the most satisfactory method. In our hands precipitation of the aluminium hydroxide *in situ* has yielded more consistent results than adsorption on a column of alumina (Lund, 1949; von Euler and Orwén, 1955). It is necessary, however, to work with a large volume of urine, otherwise high local concentration of alkali during the neutralization process is liable to destroy a high proportion of the catecholamines present.

Since, with the method described in this paper, adrenaline gives readings some 30% higher than corresponding amounts of noradrenaline our figures are liable to be too high in proportion to the amount of adrenaline present in the extracts. In the case of normal urines the adrenaline fraction of the catecholamines amounts to not more than 16% of the total (von Euler, Hellner-Björkman and Orwén, 1955) and serious errors are unlikely. In some cases of phaeochromocytoma the proportions of adrenaline are much greater, some 50–60%, but, for clinical purposes, combined estimation of catecholamines as noradrenaline has no disadvantage.

Regarding the specificity of the fluorimetric method, according to Lund (1949) at least one hydrogen atom must be attached to the α - and β -carbon atoms of the side chain for the formation of fluorescent oxidation products. Of substances satisfying this condition adrenaline, noradrenaline, and DOPA (3:4-dihydroxyphenylalanine) alone are likely to be found in biological materials. Adrenaline and noradrenaline, R_F values 0.36 and 0.28 respectively, occur closely together, while DOPA, R_F value 0.19, would not be included by the method described. The presence of DOPA in phaeochromocytomata has recently been reported by Weil-Malherbe (1956), but it has not been detected in the urine. The other substance excreted in increased amounts in phaeo-

chromocytoma, 3-hydroxytyramine, R_F value 0.39, does not give a fluorescent compound on oxidation with ferricyanide. We may conclude, therefore, that the specificity of the method for adrenaline and noradrenaline is of a high degree.

Summary

A fluorimetric method for the clinical estimation of the total combined adrenaline and noradrenaline in urine, based on the ferricyanide oxidation method of von Euler, is described.

The standard error of a single determination was $\pm 1.9 \mu\text{g.}$ per 500 ml. urine on amounts of 15 to 40 $\mu\text{g.}$ Recovery of noradrenaline added to urine was 92%, S.D. ± 9 .

Comparison of results obtained by bio-assay with those of fluorimetry showed no significant difference.

Values are given for the total excretion of adrenaline and noradrenaline in a series of normal and hypertensive subjects; there was no difference between the two groups, the range of excretion being 30–150 $\mu\text{g.}$ per day.

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Addendum

Since writing this paper the number of normal subjects examined has been brought to 25. For the entire group the daily excretion of catecholamines as noradrenaline ranged from 25 to 150 $\mu\text{g.}$, mean 82 $\mu\text{g.}$ (S.D. ± 29).

Seventy-five hypertensive subjects, in the majority of whom a phaeochromocytoma was suspected, have been examined. In four only was the excretion of catecholamines as noradrenaline $>150 \mu\text{g.}$ per 24 hours. Of these, one, who excreted 1,240 $\mu\text{g.}$, had had a phaeochromocytoma removed and was known to have another; in the second, excreting 2,600 $\mu\text{g.}$, a tumour was found and removed, while in a third patient, whose catecholamine excretion was 185 to 270 $\mu\text{g.}$, bilateral exploration of the adrenals was unsuccessful. The fourth patient, exhibiting values of 110 to 195 $\mu\text{g.}$, was not surgically explored.

Phaeochromocytomata associated with noradrenaline excretions of 180 to 300 $\mu\text{g.}$ per 24 hours have been reported by Engel and Euler (1950), Lund (1952), Burn (1953), and Goldenberg *et al.* (1954).

REFERENCES

- Burn, G. P. (1953). *Brit. med. J.*, **1**, 697.
- Engel, A., and Euler, U. S. von (1950). *Lancet*, **2**, 387.
- Euler, U. S. von (1948). *Arch. int. Pharmacodyn.*, **77**, 477.
- (1952). *Scand. J. clin. Lab. Invest.*, **4**, 254.
- and Floding, I. (1955a). *Acta physiol. scand., Suppl.* 118, p. 45.
- (1955b). *Ibid.*, Suppl. 118, p. 57.
- and Hellner, S. (1951). *Ibid.*, **22**, 161.
- Hellner-Björkman, S., and Orwén, I. (1955). *Ibid.*, Suppl. 118, p. 10.
- and Orwén, I. (1955). *Ibid.*, Suppl. 118, p. 1.
- Goldenberg, M., Serlin, I., Edwards, T., and Rapport, M. M. (1954). *Amer. J. Med.*, **16**, 310.
- Holton, Pamela (1949). *Nature (Lond.)*, **163**, 217.
- Holtz, P., Credner, K., and Kroneberg, G. (1947). *Arch. exp. Path. Pharmacol.*, **204**, 228.
- Lund, A. (1949). *Acta pharmacol. (Kbh.)*, **5**, 231.
- (1950). *Ibid.*, **6**, 137.
- (1952). *Scand. J. clin. Lab. Invest.*, **4**, 263.
- Shaw, F. H. (1938). *Biochem. J.*, **32**, 19.
- Shepherd, D. M., and West, G. B. (1953). *Nature (Lond.)*, **171**, 1160.
- Weil-Malherbe, H. (1956). *Lancet*, **2**, 282.
- and Bone, A. D. (1952). *Biochem. J.*, **51**, 311.
- (1953). *Lancet*, **1**, 974.
- (1954). *Biochem. J.*, **58**, 132.