DIFFERENTIAL ESTIMATION OF ADRENALINE, NOR-ADRENALINE IN ADRENAL GLAND AND URINE BY COLORIMETRIC METHOD

By

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Various colorimetric methods have been described for estimation of adrenaline using different chemical reagents such as potassium persulphate (1) and arsenomolybdate reduction (2) but none of them have been developed for differential estimation of adrenaline and nor-adrenaline, a serious limitation as both of these amines are usually present in the body together in varying proportion. Differential estimation by conversion to adrenochrome (3) at different pH is quite specific but suffers from sensitivity limitation (20—200 *mcg.*) Spectrophotofluro-metric method (4), the most specific and sensitive technique, is extensively being utilized for differential estimation, but involves very expensive instrumentation and very few laboratories can afford to have it.

Adrenaline has been estimated in adrenal gland by Ghosh *et al.* (5) based on reduction of alkaline phosphotungustate reagent (Folin's reagent) (6). Other substances such as ascorbic acid, cysteine, uric acid, glutathione and adrenochrome also reduce Folin's reagent. Ghosh *et al.* (5) could succeed in eliminating interference by most of these substances but the results apparantly accounted for total catacholamines, otherwise the method proved to be quite specific and sensitive.

While working with Folin's reagent at initial stages according to the method of Ghosh *et al.* it was observed that rise of temperature has untoward effect on colour intensity. Addition of Sod. hydroxide to a solution at room temperature in methodology increases the temperature of the reaction mixture by about 5°C which decreases the colour intensity. Lowering of temperature of the reaction mixture further increases the intensity of colour without much effect on the blank. The colour intensity with adrenaline and nor-adrenaline is about 10—15 times to that produced by equal amount of adrenochrome.

Initial purification of the biological extract by adsorption at pH 8.5 on alumina and elution into an acidic medium removes most of the interfering substances except ascorbic acid which is adsorbed at pH 8.5 Interference by ascorbic acid could easily be eliminated by Sod. bicarbonate treatment of purified extrct at low temperature. Differential conversion of adrenaline and nor-adrenaline to chromes at different pH and low reactivity of chromes with the reagent could well serve the basis for differential estimation of catacholamines. Sod. hydroxide treatment destroys catacholamines whereas it has no effect on other substances present in purified extract which may give colour with reagent.

In the present communication, a chemical method of reasonable sensitivity and reliability for differential estimation of adrenaline and nor-adrenaline in adrenal gland and urine is presented based on above explanation.

MATERIALS AND METHODS

Reagents :—Aluminium oxide (Acid washed), 0.5 N. Trichloracetic Acid, 0.2 N HCl, 10N H_2SO_4, Dil. acetic acid, 10% Sod. bicarbonate, 5% Sod. hydroxide, Folin's reagent, standard solution of adrenaline, nor-adrenaline, adrenochrome and ascorbic acid, 0.1N iodine in alcohol, solvent ether.

Colour concentration curves for adrenaline, nor-adrenaline, ascorbic acid and adrenochrome: Dilute solution 10 *mgc/ml.* of l-adrenaline, nor-adrenaline, ascorbic acid were prepared in 0.2 N HCl. Adrenochrome solution was prepared in distilled water. Aliquots containing 0.5, 1, 2, 3, 4, 5 *mcg.* of each in 0.5 *ml.* were prepared. Each aliquot was cooled to 15°C followed by 0.25 *ml.* of Folin's reagent, 0.5 *ml.* of 10% cold Sod. bicarbonate and 1.2 ml. of 5% cold Sod. hydroxide solution. The colour developed at 15°C was read within half a minute on Gallon Kamp Photo-electric colorimeter as Ex100 against reagent blank at 700 *mμ*. The results are represented in Fig. 1. The effect of temperature on colour intensity is shown in Fig. 2.

Extraction and purification of catacholamines from adrenal gland and urine :

(*a*) The adrenal gland of rat was homogenized with 5 *ml.* of 0.5 N trichloracetic acid in glass tissue grinder submerged in ice bath for 5 minutes. The homogenate was centrifuged to

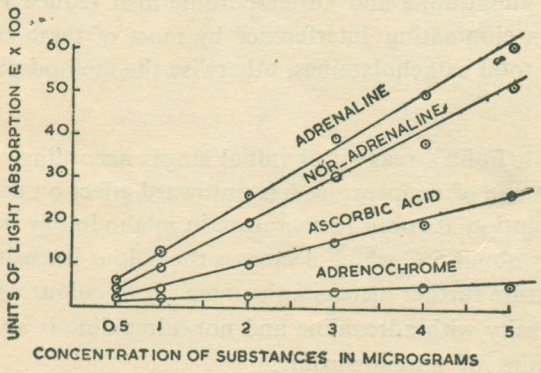

Fig 1

get clear supernatant fluid which was adjusted to 10 *ml.* with distilled water. It was then transferred to 25 *ml.* beaker containing 400 *mg.* of alumina. The mixture was brought and maintained at pH 8.5 with Sod. Hydroxide for 5 minutes with the help of pH meter. After this, stirring was stopped, the alumina quickly settled and supernatant was filtered off to avoid loss of alumina. The alumina containing adsorbed catacholamines was washed with distilled water four times, pouring supernatant liquid every time through the same filtering assembly, trans-

ferring alumina in last washing. The alumina was drained off under vaccum, and transferred quantitatively to glass stopper test tube and catacholamines eluted by vigorous shaking with 5 *ml.* of 0.2 N HCl. for 15 minutes. Acid elute was filtered to get a clear solution.

(*b*) 100 ml. of freshly collected humanurine was made 0.5 N with strong solution of sulphuric acid (10N), boiled for 10 minutes, cooed to about 4°C and centrifuged to get clear supernatant fluid. It was transferred to a beaker containing 1 *gm.* of alumina and was pocessed as for adrenal gland. The absorbed catacholamines were eluted with 3 *ml.* (used in 2 portions to improve recoveries) of 0.2 N HCl.

Aliquots of acid elutes were processed as below :—

(*a*) 0.5 *ml.* of acid elute was cooled to 15°C, 0.5 *ml.* of bicarbonate solution was slowly added from sides. The tubes were kept at room temperature for 30 minutes. Then pre-determined volume of dilute acetic acid was added to bring the pH to 3.5, cooled to about 15°C followed by 0.25 *ml.* of Folin's reagent, 1.2 *ml.* of previously cooled solution of Sod. hydroxide and the colour developed was read within half a minute.

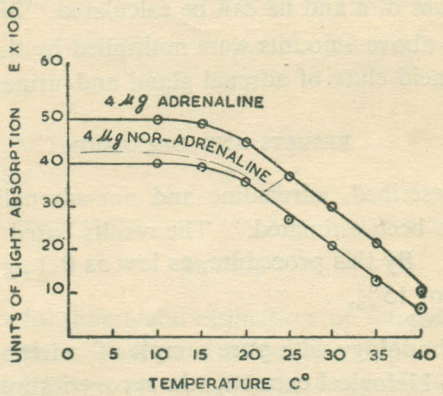

Fig 2

(*b*) After addition of acetic acid to another 0.5 *ml.* of aliquot to bring the pH to 3.5 as under (*a*), 0.1 *ml.* of 0.1 N I_2 solution was added. After 10 minutes excess of iodine was completely extracted by ether and aqueous layer was quantitatively separated by specially designed micro-separating funnel. The mixture was cooled to about 15°C and then 0.25 *ml.* of Folin's reagent and 1.2 *ml.* of cold Sod. Hydroxide solution were added.

(*c*) To another 0.5 *ml.* of aliquot, 0.5 *ml.* of sodium bicarbonate and 1.2 *ml.* of sodium hydroxide was added. After keeping for 30 minutes at room temperature, the contents were cooled to 15°C, acetic acid and Folin's reagent were then added. This served as a blank.

Calculations for differential estimation of adrenaline and no-adrenaline

$$\text{Caliberation factor for adrenaline } i.e. \quad \frac{\text{colorimetric reading}}{\mu g \text{ of adrenaline}} = m.$$

$$\text{Caliberation factor for nor-adrenaline } i.e. \quad \frac{\text{colorimetric reading}}{\mu g \text{ of nor-adrenaline}} = n.$$

pa = percentage of adrenaline not oxidized at pH 3.5 = 25% (20—25%)

pna = percentage of nor-adrenaline oxidized at pH 3.5 = 10% (8—12%)

Colorimetric reading of aliquot before I_2 treatment = X

Colorimetric reading of aliquot after I_2 treatment = Y

a = amount of adrenaline in the aliquot of acid elute

na = amount of nor-adrenaline in the aliquot of acid elute

$$m \times a + n \times na = X \quad (A)$$

$$m \times a \times pa + n \times na \times \frac{100 - pna}{100} = Y \quad (B)$$

The values for factors m, n, pa and pna in equations A & B were calculated with pure samples of catacholamine with each set of experiment. By substituting the various values in equations A & B, the values of a and na can be calculated. Since 0.5 *ml.* of aliquot of acid elute was generally used, the above amounts were multiplied by the factor to get total weight of catacholamines in original acid elute of adrenal gland and urine.

RESULTS AND DISCUSSION

By the procedures described, adrenaline and nor-adrenaline contents of rat's adrenal gland and human urine have been estimated. The results based in quadruplicate observations are given in Tables I and II. By this procedure, as low as 0.1 μg of individual amine could be measured with an accuracy of 15%.

The recoveries in methodology with pure sample of adrenaline and nor-adrenaline were 95% whereas when added to biological extracts, the recoveries were observed to be about 80%. The results are presented in Table III. Although the method can least be compared in sensitivity and specificity with fluorometric analysis, yet the method is quite simple and results are quite reproduceable. Hence it can be relied upon in absence of facilities for fluorometric analysis.

TABLE I

Adrenaline and Nor-adrenaline Contents of Fresh Rat's Adrenal Gland $\mu g/gm$)

Rat No.	*Adrenaline*	*Nor-adrenaline*
1	400	125
2	365	140
3	430	160
4	600	155

TABLE II

Adrenaline and Nor-Adrenaline Contents of Human Urine (μg/100 *ml.*)

Sample No.	*Adrenaline*	*Nor-Adrenaline*
1	1.2	6.6
2	3.3	10.2
3	2.8	9.0
4	2.0	12.0

TABLE III

Recoveries of Adrenaline and Nor-Adrenaline Added to 100 *ml. of Urine* (μg/100*ml.*)

Urine sample No.	*Added*		*Estimated*	
	Adrenaline	*Nor-Adrenaline*	*Adrenaline*	*Nor- Adrenaline*
1a	..	..	2.5	7.0
1b	5.0	5.0	6.6	10.2
2a	..	..	3.9	10.0
2b	9.0	9.0	10.9	16.8
3a	...	...	1.5	9.3
3b	2.0	2.0	2.9	10.2

SUMMARY

A colorimetric procedure for differential estimation of adrenaline and nor-adrenaline in adrenal gland and urine is described based on alkaline reduction of Folin's reagent. Differential oxidation of catacholamines at different pH and low reactivity of chrome with the reagent has been made use of for estimating amount of each amine in the mixture. As low as 0.1 μg of individual amine could be estimated with an accuracy of 15%. Recoveries of added amines were about 80%.

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