VI. THE ESTIMATION OF ADRENALINE

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REFERENCES will be found in the paper by Barker *et al.* [1932] to numerous colorimetric methods for estimating adrenaline. Since then other methods have been described by Viale [1933], Kobayashi [1935] and Whitehorn [1935], most of which are applicable only to concentrations >1 μg./ml. and are too unspecific for application to extracts of tissues other than the suprarenals. Whitehorn claimed to estimate adrenaline in blood in a concentration of 0·02 μg./ml. The present paper describes a modification of Whitehorn's method which is simpler and more sensitive.

The method depends on the fact that adrenaline reduces arsenomolybdic acid with the formation of a blue colour. It has been found that brief preliminary treatment of adrenaline with alkali in the presence of oxygen increases the colour considerably. If the treatment with alkali is prolonged the colour is decreased. Adrenaline is known to be oxidized under these conditions, and apparently one of the products of oxidation is a more active reducing agent than adrenaline itself; this product is itself destroyed by longer exposure to alkali. The nature of this product is unknown. A series of substances allied to adrenaline, including adrenalone has been tested, but none is the product in question since none produced as much colour as adrenaline itself. *p*-Sympatol was the only substance which resembled adrenaline in giving increased colour after alkali treatment; it was also the only substance whose side chain was identical with that of adrenaline. These facts suggest that this particular property of the side chain of adrenaline is highly specific, and that if the reducing power of a tissue extract be increased by treatment with alkali the observation can be taken as evidence that the extract contains adrenaline or some other phenol with the same side chain.

Arsenomolybdic acid is reduced by a number of substances besides phenols, such as ascorbic acid, reduced glutathione, eserine, various polyhydroxy and thiol compounds and in general $\Delta^{1,2}$-diols. To eliminate interference by such substances. Whitehorn employed silicic acid which adsorbs the adrenaline, but not the other compounds. He did not, however, succeed in recovering more than 50 % of the adsorbed adrenaline, and he multiplied his results by a factor in order to compensate for the loss at this stage. It has been found more convenient to adsorb the adrenaline on a specially prepared aluminium hydroxide, which is afterwards dissolved in the reagents of the test so that the adrenaline is recovered completely. The optimum *p*H for the adsorption of adrenaline is between 8·0 and 8·5; on the acid side of *p*H 7·0 no adsorption takes place. If an extract is first shaken with $Al(OH)_3$ at *p*H 4 glutathione, and possibly other substances, are removed. Ascorbic acid is not adsorbed at either alkaline or acid reactions.

A test has therefore been devised in which the fraction adsorbed at *p*H 8·5, but not at *p*H 4, is heated with alkali and mixed with the arsenomolybdic reagent. This test is probably at least as specific as that described by Whitehorn.

It is simpler and more sensitive, and there is no need to compensate for any loss of adrenaline. In addition a very specific qualitative test is described, which depends on the effect of alkali.

Experimental

Reagents

(1) *Arsenomolybdic acid.* 60 g. of crystalline sodium molybdate and 10 g. of sodium arsenate are dissolved in about 250 ml. of water and filtered. The filter is washed and to the filtrate and combined washings are added 5 ml. of bromine water and water to 500 ml. For use, 100 ml. of this solution are mixed with 8 ml. of conc. H_2SO_4.

(2) *1:1 Sulphuric acid.* A mixture of equal volumes of conc. H_2SO_4 and water.

(3) *Sulphite and sulphurous acid.* 10 g. of $Na_2SO_3,7H_2O$ are dissolved in 50 ml. of water. This must be prepared every 2 days. Before use 2 ml. are added to 14 ml. of 1:1 H_2SO_4. This must be prepared every half hour.

(4) 4% *Sodium hydroxide.*

(5) *Approx.* N *sulphuric acid.*

(6) *Phenolphthalein.* Dissolve 0·1 g. in 100 ml. of *N*/100 NaOH. It is not permissible to use alcohol. This solution will keep about 3 days.

(7) *Aluminium hydroxide.* 25 g. of pure potassium alum are dissolved by heating in about 200 ml. of water and the solution cooled to about 20°. 5 g. of NaOH in 20 ml. water are added slowly with stirring. The precipitate is filtered and washed several times and suspended in about 100 ml. of water, so that it just flows freely from a pipette. Each batch should be soluble in 4% NaOH, and should be tested for its efficiency in adsorbing and liberating adrenaline. Owing to some action of the $Al(OH)_3$ on adrenaline the apparent recovery is usually 110%.

(8) *Standard adrenaline.* A dilution of 1 : 10,000 is prepared in approx. *N*/100 H_2SO_4. This will keep several days. It is diluted to 1 : 10 million, etc. with *N*/100 acid as required. The diluted standard will keep several hours. The stability is improved if a little glycine is added.

Procedure

The solution to be tested, which should contain between 0·1 and 0·5 μg. of adrenaline (though 0·04 μg. can be detected) should be neutral or faintly acid. Blood should be run into an equal volume of 10% trichloroacetic acid as rapidly as possible after removal from the body. If it is kept for a few min. a substance is formed which is indistinguishable in this test from adrenaline. Other tissues are dropped into trichloroacetic acid (at least 1 ml. per g. of tissue) and then cut up finely. After 30 min. the mixture is filtered and the residue is well washed with trichloroacetic acid. The filtrate or a suitable aliquot is placed in a centrifuge tube, 2 drops of phenolphthalein reagent are added and it is then carefully neutralized with 4% NaOH. One drop of *N* H_2SO_4 is added (approx. *p*H 4·0), then 2 ml. of the suspension of $Al(OH)_3$. The solution is shaken and centrifuged (3000 r.p.m.) for 2 min. The supernatant fluid is poured into another tube, and 1 ml. $Al(OH)_3$ per 5 ml. of solution and 1 drop of phenolphthalein are added. Caustic soda is now added drop by drop with shaking until the solution is just distinctly pink (*p*H 8·5). The solution is shaken and centrifuged as before. The supernatant fluid is discarded, and about 3 ml. of water, made just alkaline to phenolphthalein with NaOH, are poured on to the precipitate; it is centrifuged

and the fluid discarded. 2 ml. of water and 0·35 ml. of 4% NaOH are added to the $Al(OH)_3$, which should now go into solution.

The solution is kept for 2 min. and then 2 ml. of the H_2SO_3 reagent are added and the mixture poured into a tube in a boiling water bath which contains 0·7 ml. of the arsenomolybdic acid which has been heating for 5 min. After exactly 5 min. the tube is removed and placed in a beaker of cold water. The volume is made up to 5·5 ml. and the colour estimated in a colorimeter 15–20 min. later. The colour does not fade but remains constant for at least an hour and then tends to increase slowly.

A blank is prepared by taking the same volume of $Al(OH)_3$ suspension as has been used in the test, adding 2 drops of phenolphthalein, making just alkaline with 4% NaOH and centrifuging. The supernatant liquid is discarded, 2 ml. of water, and 0·35 ml. of 4% NaOH are added to the $Al(OH)_3$, then the H_2SO_3, etc. as before.

A standard blue solution is prepared by subjecting 2 ml. of an appropriate solution of adrenaline to the second, but not necessarily the first, adsorption with $Al(OH)_3$ and subsequent treatment with alkali, sulphite and arsenomolybdic acid as described above. A Leitz two-stage colorimeter with a standard grey solution and filter No. 9 was used for comparing the solutions, but no doubt a simpler instrument could be used. The colour due to the blank must be subtracted both from that due to the unknown and that due to the standard.

The curve shown in Fig. 1 shows the relation between the amount of adrenaline and the colour, after subtracting the colour corresponding to the blank test. This curve is approximately linear for small, but not for large, amounts of

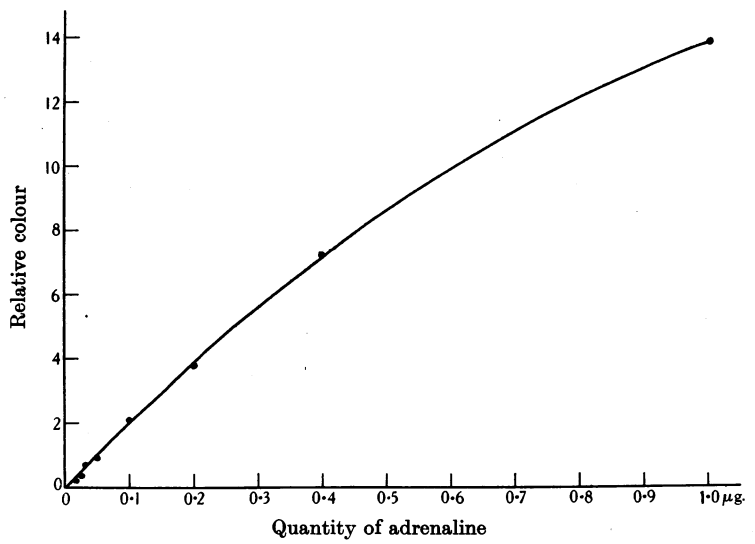


Fig. 1. Colour, in terms of the standard grey solution (using filter No. 9), when adrenaline is subjected to the complete test.

adrenaline. The simplest method of calculating the result of a test would be to read off the amount of adrenaline directly from a curve such as that shown in the figure. This method has been found unreliable because, although constant results have been obtained on any one day, unexplained variations in the position of the curve have occurred when the test has been repeated on different days.

It has therefore been necessary to compare the colour due to unknown solutions with that due to adrenaline solutions treated with the same reagents on the same day. It is found that if the total quantity of adrenaline is less than 0·4 μg. and the ratio of intensities of colour in the two tubes is less than 2, the error introduced by assuming that the colour is proportional to the amount of adrenaline is less than 10%. For some purposes this is accurate enough. Greater accuracy can be obtained by using as standard the colour due to an amount of adrenaline more nearly equal to that contained in the test of the unknown solution, the colour of which is constant for at least an hour.

The smallest absolute amount of adrenaline which can be estimated is about 0·04 μg. and the lowest concentration in which this amount has been estimated was 1 in 5×10^8. The standard deviation of a single observation was estimated as about 0·015 μg.

Specific test

This depends on the increase of colour due to alkali. The solution to be tested is divided into two. One half is treated as above and the other in the same way except that the 0·35 ml. of 4% NaOH which is used to dissolve the $Al(OH)_3$ after the second adsorption is replaced by 0·35 ml. of water containing 1 drop of N H_2SO_4. When the reduction is due to 0·04 μg. or more of adrenaline the ratio of the colour given by the alkali-treated half of the solution to that given by the untreated half is from 2 to 3·5. With smaller quantities the ratio falls.

Table I shows the concentrations of a number of substances required to give a total colour equivalent to that given by adrenaline (1 μg./ml.). These estimates were prepared by testing the solutions of the substance directly without adsorption. The proportional increase of colour when adrenaline was treated with alkali was found to be 5 whereas the ratio was only 3·5 when the two fractions of adrenaline were both adsorbed by the method described above. This difference is apparently due to the fact that the small amount of alkali necessary for adsorption changes the adrenaline slightly so as to increase the colour, thus reducing the apparent effect of the larger quantity of alkali.

Table I also shows that catechol derivatives are active in low concentrations, and that small changes in the side chain abolish the effect of alkali. Sympatol was the only other substance tested which contains the same side chain as adrenaline and the ratio was about the same as that for adrenaline, though it was necessary to employ high concentrations to obtain any appreciable colour. All other substances tested in the series gave ratios of 1 or less. On the other hand, the small amount of colour given in blank tests was found to be increased by alkali in the ratio of 1·1–1·5. Ratios of 2 or over can therefore be used as evidence for the presence of adrenaline.

Results with tissue extracts

Recovery of added adrenaline. Adrenaline was added to rabbits' blood to make a final concentration of 0·1 μg./ml., and it was found, by subtracting the colour due to the blood from the total colour that the recovery was 75–80%. A small loss occurred during the extraction with trichloroacetic acid, since if the adrenaline was added after this stage the recovery was 99%.

Comparison with the biological test. Extracts of suprarenal glands were prepared with trichloroacetic acid, and the adrenaline content was estimated by the test described above and also by comparison of the pressor effect on spinal cats with that of a standard solution of adrenaline. Table II shows satisfactory agreement between the two tests.

Table I. *Showing the specificity of the test*

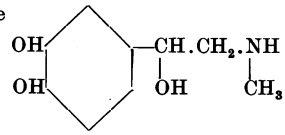
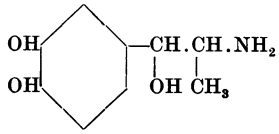
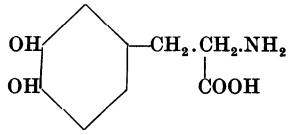
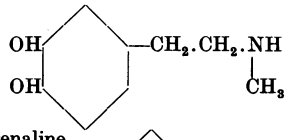
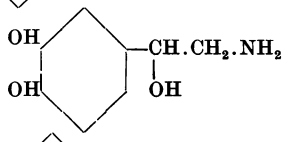
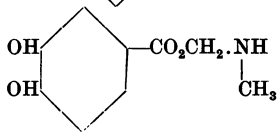
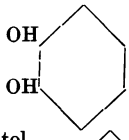
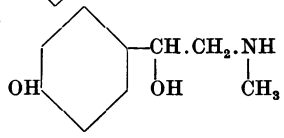
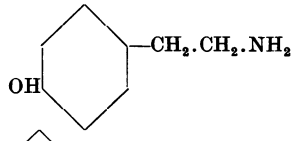
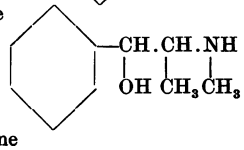
Substance	Concentration (μg./ml.) giving equivalent colour after alkali treatment	Proportional increase of colour due to alkali
Adrenaline 	1	5
Corbasil 	12	No change
Dopa 	12	,,
Epinine 	13	,,
nor-Adrenaline 	16	,,
Adrenalone 	30	,,
Catechol 	100	Diminishes
p-Sympatol 	2000	5
Tyramine 	No colour	—
Ephedrine 	,,	—
Glutathione	500	No change
Ascorbic acid	500	Diminishes
Glyceraldehyde	1000	,,

Table II

Animal	Adrenal content of suprarenal μg./g. Cat blood pressure	Adrenal content of suprarenal μg./g. Colorimetric	Proportional increase due to alkali
Dog	450	400	3·2
Rabbit	150	160	3·1
	250	270	3·4
	600	600	3·4
	1320	1500	—

Other tissues. The results of the application of the test to other tissues are shown in Table III.

Table III

Tissue		Apparent adrenaline content μg./g.	Proportional increase due to alkali
Frog:	heart	0·30, 0·40, 1·0, 1·2	2·5, 2·2
Rabbit:	prostate	0·34, 0·40	2·5, 2·0
	blood	0·050, 0·052, 0·060	1·9, 1·7, 1·5
	intestine	0·033, 0·033	1·0
	heart	0·015, 0·016, 0·017, 0·023, 0·04	1·3, 1·4, 1·5
	liver	0·019, 0·04	1·0
	kidney	0·021, 0·022	1·0, 1·0
	stomach	0·01, 0·019, 0·025	1·0, 1·0
Man:	blood	0·016, 0·020	1·2

The results with frog's heart and rabbit's prostate show rough quantitative agreement with those of Loewi [1936] and Euler [1934] who have found evidence of the presence of adrenaline in these tissues. The increase of colour with alkali confirms the view that the active substance actually is adrenaline.

The other tissues tested certainly did not contain comparable quantities of adrenaline and, in particular, the rabbit's heart contains much less than the frog's heart. In most of the other tissues the blue colour was not increased by alkali and was therefore probably not due to adrenaline. In some cases there was a small increase of colour due to alkali, and though it would, of course, be unwise to assume, without further evidence, that this was due to the presence of adrenaline, this probability is suggested by the results. The presence of such quantities of adrenaline in normal blood serum or plasma appears to be excluded by experiments such as those of Trendelenburg [1923] and Schlossmann [1927].

This fact does not prove that the results obtained in these tests were not due to adrenaline. The blood was collected from the head wound after a rabbit had been killed with a "humane killer" and it is probable that adrenaline is liberated in these conditions. Furthermore, adrenaline passes rapidly into the blood cells [Bain *et al.* 1936] and would therefore be present in extracts of whole blood and not in the serum or plasma.

The results of Table III illustrate the limitations of the method for the estimation of tissue extracts. The amounts of substances other than adrenaline which reduce the arsenomolybdic reagent are equivalent to about 0·01–0·04 μg. of adrenaline per g. of tissue (rabbit liver, intestine, kidney, stomach). Consequently the limit of the method for tissue extracts is probably of the order of 0·1 μg. adrenaline per g.; even results of this order must be interpreted with caution.

SUMMARY

1. A colorimetric method is described which will estimate 0·04 μg. of adrenaline with a standard error of about 0·015 μg.

2. When applied to suprarenal extracts this test gives results agreeing with biological estimates.

3. A specific test is described by which the colour due to adrenaline can be distinguished from that due to other substances.

4. Adrenaline can be estimated in tissues provided that the concentration exceeds 10^{-7}. If the concentration is lower than this, the test gives a result which must be interpreted with caution.

I wish to express my thanks to Prof. J. H. Gaddum for his continued interest and advice throughout the course of this work.

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