LXXVI. OBSERVATIONS ON THE CHEMICAL DETERMINATION OF ADRENALINE

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IN the routine estimation of adrenaline in suprarenal or other tissue extracts none of the chemical or physiological properties so utilized can be regarded as specific to adrenaline. Much work has been done in recent years to decide which of these is least liable to error. Notable discrepancies in the results obtained by a variety of physiological methods have been recorded by the Tokio School for suprarenal extracts from different species of animals [Sugawara, 1928; Watanabe & Sato, 1928; Aomura, 1929; Kojima *et al.* 1932; Sato & Inaba, 1932]. Such differences are ascribed to the presence of other physiologically active substances which affect different methods in a characteristic way. The possibly serious effects on the pressor assay, for example, of choline and acetylcholine (the distributions of which in the bovine adrenal gland are given by Feldberg & Schild [1934] have been well illustrated by Hunt & Taveau [1911]. The actual operation of this factor however in ordinary extracts is doubtful [Sugawara, 1928].

Of the many chemical (colorimetric) methods described, the two now in general use depend either on (a) the oxidation of adrenaline preferably by iodine or mercuric chloride to a "characteristic" red pigment, or (b) the reduction of Folin's uric acid reagent to a blue derivative. In the first of these, the red colour is also given in comparable intensity by allied compounds such as dopa and epinine [Ewins, 1910; Maiweg, 1922]. Since there is no satisfactory evidence for the presence of such compounds in the adrenal gland the agreement between the oxidation method and the pressor assay in this and other papers [Euler, 1933; Schild, 1933; Devine, 1936] suggests that both yield substantially correct results. On the other hand Folin's reagent is sensitive to a much wider range of compounds [Levine & Burns, 1922; Barker *et al.* 1932] including uric acid, physiologically inactive oxidation products of adrenaline [Maiweg, 1922] and ascorbic acid and its application has given rise, more than any other colorimetric method, to diverse results. Satisfactory agreement with the pressor assay was observed by Folin *et al.* [1912] and by Stewart & Rogoff [1916] for acid and neutral adrenal extracts; abnormally high values for the Folin method by Lewis [1916] in foetal bovine glands, by Maiweg [1922] in partially oxidized solutions, by Frowein [1923] in the glands of normal and, more especially, "sugar-punctured" animals, and also by Barker *et al.* [1932] and by Watanabe & Sato [1928]; while on the other hand errors in the reverse direction have been reported by Sydenstricker *et al.* [1914] and Schild [1933]. According to Kojima *et al.* [1932] the error in excess shown by the Folin method is due to a reducing substance relatively much more concentrated in the cortex than in the medulla. The recent identification of this material as ascorbic acid makes it possible to investigate the effect quantitatively. Investigation by Lewis [1916] of the error due to uric acid was limited to foetal glands, the figures for which are not necessarily applicable to the adult tissue.

The experiments described in the present paper were undertaken to correlate those of previous workers by assessing the combined errors in the Folin assay of bovine suprarenal extracts due to ascorbic and uric acids and comparing the corrected values with those obtained by other accepted methods.

METHODS

The Folin estimation. The colour development of Folin's reagent depends on the temperature [Baker & Marrian, 1927] but chiefly on the final pH of the solution. The acid reagent is readily reduced at room temperature by both uric and ascorbic acids without addition of alkali, but not by adrenaline which, even under neutral conditions, yields a slowly developing violet instead of a blue colour. This point is emphasized by Barker *et al.* [1932] whose modification designed to give more consistent results than the original method has been used throughout this work. Instead of saturated Na_2CO_3 a definite excess of NaOH is added according to the initial acidity of the reagent. The latter was prepared from an "A.R." sample of sodium tungstate freed from molybdenum according to the directions of Folin & Trimble [1924] to eliminate the interference of phenolic compounds which might be present in the extracts, and 2 ml. were equivalent to 2.84 ml. *N* NaOH. The standard colour solution was therefore made up as follows: 2 ml. reagent, 4 ml. adrenaline (1 : 100,000), 4.5 ml. *N* NaOH and similarly with the extracts, the acidity of which was negligible after suitable dilution.

Uric acid estimation. A suitable aliquot of each extract was made just alkaline to Congo red and the uric acid precipitated by a 50 % excess of 5 % silver lactate. After 5 min. in the dark the mixture was centrifuged and the residue washed twice with water and finally dissolved by adding 2 ml. 5 % LiCl, 3 ml. 5 % NaCN and 5 ml. H_2O . The uric acid was then estimated by the Folin method by comparison against a similarly constituted standard. The stock solution was made according to Benedict & Hitchcock [1915] from a freshly recrystallized sample.

Ascorbic acid estimation. The extracts were titrated against a standard indophenol solution; pure ascorbic acid solutions against iodine. In order to prevent loss of ascorbic acid from extracts by oxidation during the experiment each sample was diluted and treated with the Folin reagent immediately following the estimation of the ascorbic acid in another aliquot.

RESULTS

Ascorbic acid error

The colour-concentration curve of medullary and cortical extracts was first obtained by determination of the apparent adrenaline content after addition of known amounts of ascorbic acid. The extracts were prepared with 1 % trichloroacetic acid and deproteinized by heating for a short time to boiling. As both curves are linear, as illustrated in Fig. 1, titrimetric estimation of the ascorbic acid in an extract allows us to calculate the error provided that we know the ascorbic acid-adrenaline colour ratio. This is given as 3 : 8 (0.375) by Euler *et al.* [1932] using the original Folin method and the value was confirmed on different solutions at two different pH values by the modified version (0.373, 0.389, 0.371, 0.369, 0.379; mean value 0.376).

In Table I (Exps. I and II) the corrected Folin estimates for different batches of glands are compared with those obtained by the iodine oxidation method [Schild, 1933], the nature of the extracts being further confirmed in some cases

by estimation of "total catechol" substances [Devine, 1936] or the pressor activity on a pithed cat.

Fig. 1. Colour-concentration curves showing effect of added ascorbic acid on the apparent adrenaline content (Folin estimate). Points A (medulla) and B (cortex) represent the average composition of the extracts.

Table I. *mg. material per g. of fresh tissue*

Exp. no.	Ascorbic acid	Adrenaline equiv.	Apparent Folin	Corrected Folin	Adrenaline (iodine)	Total catechol	Pressor assay
I. Medulla:							
1	0.58	0.22	9.32	9.10	8.89	10.0	—
2	0.58	0.22	10.5	10.3	10.3	11.6	—
3	0.59	0.22	8.97	8.75	—	—	—
4	0.75	0.28	12.1	11.8	12.2	13.9	—
II. Cortex:							
1	0.59	0.22	0.75	0.53	0.46	—	0.46
2	0.72	0.27	1.00	0.73	0.52	—	0.47
3	0.57	0.21	0.78	0.57	0.46	—	—
4	0.45	0.17	0.78	0.61	0.45	—	—
III. Cortex:							
1	0.08	0.03	0.99	0.96	0.74	—	—
2	0.67	0.25	1.1	0.85	0.62	—	—
3	0.36	0.13	0.83	0.70	0.60	—	—
4	0.16	0.06	1.2	1.1	0.80	—	—

The following points are clear:

(1) In the medulla the ascorbic acid effect is small (about 2% in excess) as is to be expected from the relatively high concentration of adrenaline. As the corrected values are seen to compare very well with those of the iodine method, i.e. ascorbic acid accounts for the whole of the error, no further analyses were made.

(2) In the cortex the error is much greater depending on the precautions adopted to prevent *post mortem* diffusion of adrenaline from the medulla which, according to Watanabe & Sato [1928] and Kojima *et al.* [1932], is wholly responsible for the adrenaline content usually detected. In this case the correspondence of the corrected Folin with the iodine estimate was unsatisfactory, the mean ratio being 1:1.3 which was recorded again in a similar experiment (Exp. III). As the bovine cortex contains about 1.8 mg. ascorbic acid per g. [Harris & Ray, 1933; Glick & Biskind, 1935] the bulk of this must have been destroyed in the extraction process. This suggests that apart from other reducing

substances partial oxidation products of ascorbic acid not detectable by indophenol titration might reduce the Folin reagent. This point was tested as follows.

(a) The extracts, after analysis, were allowed to stand sealed at 0° until their ascorbic acid contents (indophenol titre) were negligibly small. The iodine and Folin estimations were now repeated and also the "total catechol" was estimated. The slight loss of adrenaline observed was taken into account in calculating the figures in Table II for the fresh solutions supposing that they had contained no ascorbic acid. In the second set (of Exp. III) the solutions still contained a little which was allowed for.

Table II. *mg. adrenaline per g. of fresh tissue*

	Exp. no.	Adrenaline (iodine)	Total catechol	Folin
II	1	0.46	0.71	0.65
	2	0.52	0.83	0.76
III	1	0.74	—	0.94
	2	0.62	—	0.96
	3	0.60	—	0.70

The relation between adrenaline and "total catechol" was as previously observed [Devine, 1936]. In two cases (Exp. II (1) and Exp. III (2)) the observed Folin values were still notably greater than the "corrected" values of Table II so bearing out the suggestion made. Of the remaining results which showed good agreement two were unreliable owing to difficulty in judging the end-point.

(b) As these experiments were not conclusive further evidence was obtained from solutions of pure ascorbic acid which were standardized against iodine when fresh and again after standing overnight at 0° (A). The partially oxidized solution was now compared by both iodine and Folin titrations against a fresh solution (B) and in one case by indophenol titration which was less exact.

Table III. *Estimation of partially oxidized ascorbic acid solutions*

% Oxidation		Ascorbic acid A/B		
A	B	Iodine	Folin	Indophenol
81	0	1.4	3.3	—
83	11	1.4	3.3	—
92	0	0.31	1.4	0.24

Combined ascorbic and uric acid error

The results summarized in Table III clearly show that Folin's reagent is sensitive to ascorbic acid derivatives not detectable by indophenol or iodine. Strict comparison of the iodine and Folin methods of adrenaline estimation is therefore impossible unless precautions to avoid oxidation be taken. Hence for estimation of the combined ascorbic and uric acid errors given in Table IV the extracts were made with as little delay as possible with 2% cold metaphosphoric acid as recommended by Fujita & Iwatake [1935] and Levy [1936]. Nevertheless the ascorbic content found was only about 1 mg./g. A preliminary estimate of the uric acid in 6% trichloroacetic extracts of cortex yielded a mean value of 0.12 mg./g. equivalent to only one tenth the ascorbic acid error, using the adrenaline-uric acid colour ratio of 10 : 3 [Euler *et al.* 1932]. In the phosphoric acid extracts the content was less, so that although great accuracy was not obtainable, because the precipitation of AgPO₃ necessitated the use of smaller aliquots, the figures serve the present purpose.

Table IV. *mg. adrenaline per g. of fresh tissue*

Adrenaline equivalent		Combined error	Apparent Folin	Corrected Folin	Adrenaline (iodine)
Uric acid	Ascorbic acid				
0.02	0.41	0.43	0.80	0.37	0.36
0.02	0.48	0.50	0.97	0.47	0.39
0.02	0.49	0.51	0.96	0.45	0.43
0.02	0.44	0.46	0.89	0.43	0.42

The agreement recorded is satisfactory.

DISCUSSION

The variability of the estimates by the Folin method recorded in the literature can now be ascribed chiefly to:

(a) Ascorbic acid, responsible for errors in excess, depending on the precautions taken during the extraction process and storage to prevent its destruction. In view of the protective function of ascorbic acid, it is this latter effect and probably not the destruction of adrenaline as supposed by Folin *et al.* [1912] and Baker & Marrian [1927] which causes discrepancies in estimates carried out at intervals on the same solution.

(b) The pH at which the colour is developed, particularly with respect to the reducing action of adrenaline. Failure to appreciate this point is probably responsible for the abnormally low values recorded [Sydenstricker *et al.* 1914; Schild, 1933]. These two sources of error can operate against one another so yielding results in good agreement with the pressor assay [Folin *et al.* 1912; Stewart & Rogoff, 1916].

The satisfactory agreement now recorded between the pressor assay and the iodine and Folin (corrected) estimates of adrenaline in the suprarenal cortex and medulla shows also that the unidentified catechol derivative which is also present and which does not give a red colour on oxidation has little or no pressor activity, or effect on the Folin reagent. Moreover, if any other precursor of adrenaline is present in the gland it can exist only in such small amount as lies within the experimental error of the adrenaline estimates. Otherwise it must have approximately parallel effects on all three methods of estimation and this is extremely improbable. Such claims as have been advanced rest only on discrepancies arising from doubtful methods of estimation.

SUMMARY

1. The error in excess previously observed in the Folin estimation of adrenaline in suprarenal extracts as compared with the more reliable oxidation method or pressor assay is almost quantitatively accounted for by the ascorbic and uric acid present, chiefly the former; the error in the reverse direction is probably due to a low pH. The validity of the correction is limited by the sensitivity of the reagent to partially oxidized ascorbic acid.

2. Apart from the previously observed but unidentified catechol compound, the claim that an adrenaline precursor exists in the gland is not confirmed.

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