CCXLVII. SO-CALLED VIRTUAL ADRENALINE OF THE SUPRARENAL CORTEX.

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THE close structural relationship which adrenaline bears to the two naturally occurring amino-acids, tyrosine and phenylalanine, has focussed attention on both of these substances as possible biological precursors. The claim of Halle [1906] that adrenaline is formed when suprarenal tissue is incubated with tyrosine was shown conclusively by Ewins and Laidlaw [1910] however to be based on unsound evidence. Similar negative results have also been reported by Nikolajeff [1924] on perfusion of tyrosine through the surviving suprarenal gland; by Trendelenburg [1929] who treated the pulped gland with dihydroxyphenylethyl-methylamine and also by Funk [1912] in similar experiments with 3:4-dihydroxy-phenylalanine (dopa). Heard & Raper [1933] have shown that both dopa and *N*-methyldopa are stabilized against subsequent oxidation when perfused through the surviving adrenal gland whereas when exposed to the action of tyrosinase they yield small amounts of pressor bases (probably aminoaceto-catechol and adrenalone respectively). Apart from the recent experiments of Schuler & Wiedermann [1935] which have not yet received confirmation there is then little satisfactory evidence that the synthesis of adrenaline actually takes place in the gland or that the latter contains any demonstrable precursor. It must be realized however that failure to demonstrate any such formation by minced or surviving tissue does not preclude the possibility of some change, necessary to the process as a whole, having taken place.

Another possibly significant factor is the distribution of adrenaline as between the cortex and medulla of the gland. The small fraction generally found in cortical extracts may be due in part to post mortem diffusion from the medulla and to the impossibility of effecting an absolutely "clean" separation of each type of tissue by dissection. For this reason and also because the tissues are morphologically independent little attention other than that of Abelous *et al.* [1905] has been paid to the possible role of the cortex in the elaboration of adrenaline. More recently Abelous & Argaud [1931, 1932, 1934] have concluded that adrenaline is merely stored in the medulla after it has been formed in the cortex from a parent substance "virtual adrenaline".

The purpose of the present communication is to describe attempts to reproduce these latter experiments and to study the effect of "virtual" adrenaline on the assay of suprarenal extracts.

EXPERIMENTAL.

Estimation of adrenaline.

The principal methods of estimating adrenaline are based either on (a) its oxidation to a "characteristic" red pigment, or (b) its reduction of Folin's phosphotungstate reagent.

Both of these methods are likely to give erroneously high results in the assay of suprarenal extracts if other chemically allied substances are present. Heard [1932] has tested both types of estimation with the related compounds dopa, methyl dopa and epinine using the Folin method [Folin *et al.*, 1912] and the various oxidizing agents proposed by Vulpian [1856], Comessatti [1908] and Ewins [1910]. In each case the tint and intensity of colour were identical with those given by an equimolecular concentration of adrenaline. Folin's method is liable to further error since the reagent is reduced by uric acid and other extractives including, particularly, ascorbic acid which is present in both the medulla and cortex of the gland. In the present experiments the persulphate method of Ewins as modified by Barker *et al.* [1932] has been used since a careful comparison made by them showed that this gave results most consistent with the physiological assay (pressor activity).

In order to gain additional information as to the mechanism of adrenaline formation the total amount of catechol substances also present at different stages of the process was estimated by the ammonium molybdate method of Rae [1930]. In deproteinized cortical extracts colour matching against a standard adrenaline solution is made difficult by the presence of ascorbic acid which, with molybdate, gives a similar yellow colour turning finally an intense blue after a few minutes. The error on this account is not serious provided that the solution is assayed immediately after adding the reagent. In a test experiment, minced cortical tissue of the bullock, after being shaken in air for 3 hours with 0.5% sodium carbonate, was found by indophenol titration [Birch *et al.*, 1933] to contain only 0.4 mg. ascorbic acid/g. tissue. The effect of this concentration could be ignored since the standard solution, equivalent to four times this concentration, did not itself give an effective colour with molybdate.

The hypothesis of “virtual” adrenaline.

The results of Abelous & Argaud [1931, 1932, 1934] already referred to may be summarized as follows:

(a) If the minced cortical tissue of horse suprarenal gland is shaken at room temperature for several hours in 3% Na_2CO_3 then both chemical and physiological assays show an increase in adrenaline content. Under the same conditions all the adrenaline in medullary tissue or in a mixture of medullary and cortical tissues is destroyed. These effects are not dependent on the presence of tissue and take place in deproteinized acid extracts subsequently made alkaline.

(b) A deproteinized *aqueous* extract of cortex contains much more adrenaline measured physiologically (pressor effect) than by colorimetric assay; whereas in an aerated alkaline extract the pressor response is the same as before whilst the chemical assay shows a marked increase.

Hence it is concluded that the suprarenal cortex contains very little free adrenaline but a relatively large quantity of “virtual” adrenaline which has a similar pressor activity, does not respond to colorimetric tests and is converted into adrenaline by oxidation in alkali. In the medulla the proportions of free and “virtual” adrenaline are reversed.

The first two attempts to repeat these experiments were made with the suprarenal glands of the bullock. These were de-fatted and dissected immediately on arrival in ice from a local abattoir. As noted by Schild [1933], numerous assays made in the course of this investigation indicate that elaborate precautions against the decomposition of active material are unnecessary. The tissue was minced and thoroughly mixed and samples of 10 g. medulla and 20 g.

cortex weighed into wide-necked 200 ml. bottles and each pair treated separately as follows, except in IV and V which refer to *mixtures* of 10 g. medulla and 20 g. cortex:

I. (Controls). Extracted with $N/10$ H_2SO_4 .

II and IV. Shaken for $1\frac{1}{2}$ hours in air in 0.1% Na_2CO_3 .

III and V. Shaken for 4 hours in air in 0.1% Na_2CO_3 .

At the end of the process each sample was acidified with 10 ml. N H_2SO_4 to stabilize the adrenaline then corked and kept overnight at 0° . The mixture was filtered through muslin and the residue ground with sand and re-extracted. The total crude filtrate was heated nearly to boiling and the protein removed by adjusting the pH to 5.5 (methyl red). A water-clear, almost colourless extract was thus obtained from which aliquots were removed for the estimation of adrenaline and of total catechol substances. At the same time the adrenaline content was assayed physiologically by estimating the pressor activities of the deproteinized solutions by intravenous injection into a pithed cat. The results are presented in Table I, the material responding to each test being reckoned as mg. adrenaline in the whole sample of tissue.

Table I.

Sample	Persulphate	Molybdate	Pressor assay
I. M	41	144	185
C	10	21	15
II. M	43	136	157
C	10	18	15
III. M	41	126	149
C	8	18	12
IV. (M + C)	28	146	202
V. (M + C)	26	150	185

In each Table, unless otherwise stated, M = Medulla (10 g.); C = Cortex (20 g.).

As there was doubt at this stage as to the validity of the persulphate estimation the marked discrepancy shown between the chemical and physiological assays will be discussed later. If attention is confined to the latter it will readily be seen that they in no way bear out the results of Abelous and Argaud previously discussed. If MI and CI (*acid* extracts)¹ be accepted as controls for the remaining samples (*alkaline* extracts) then it appears that the adrenaline present in the medulla does not wholly disappear, but rather is the stabilizing effect of the system emphasized. Thus after 1.5 hours' exposure to air in alkaline solution, 85% of the pressor activity still remained and only a further 5% was lost at the end of 4 hours. Conversely the activity of the cortical tissue does not show a marked rise but a fall of about 20% as in the medulla. In the samples of mixed tissue the predominant effect was the same.

As the conditions had been made less drastic so as to minimize the destructive effect of oxidation, the experiment was repeated adhering more closely to the conditions originally laid down. The treatment was therefore carried out in 3% Na_2CO_3 , to which a little octyl alcohol was added, by drawing a steady stream of air through the bottles which were immersed in a thermostat at $34-35^\circ$. The control extracts were made under hydrogen. The effect was also studied on protein-free acid extracts which were assayed before and after alkaline aeration

¹ The difference between acid and alkaline extracts is probably not significant. Cf. Table II.

thus eliminating errors due to lack of homogeneity in sampling the tissue. The treatment is summarized below and the results presented in Table II.

- I. (Controls.) Extracted with alkali under hydrogen for 4 hours.
- II. Aerated in alkali for 4 hours.
- III. Extracted with $N/100$ H_2SO_4 under hydrogen.
- IV. Extracted with water under hydrogen.
- A. Deproteinized aliquot of IIIC made alkaline and aerated.
- B. As in A with addition of 10 g. fresh medulla before aeration.

Table II.

Sample	Persulphate	Molybdate	Pressor assay
I. M	50	126	152
C	10	19	13
II. M	52	136	130
C	6	20	7
M + C	52	182	124
III. M	14	125	104
C	7	17	13
IV. M	39	119	118
C	10	11	9
A*	0	0	0
B*	—	—	40

* These analyses are referred to the whole of IIIC, as "aliquots". Chemical analysis of B was impossible on account of the deep colour of the solution. As further decomposition appears to have taken place before the physiological assay could be made the value obtained represents only a minimum figure.

The pressor assays of samples I and II indicate substantially the same results as before save that the failure of the cortical tissue to general adrenaline is even further emphasized since after 4 hours' treatment the destruction in the cortex (44%) was three times as great as in the medulla and nearly twice as much as in a mixture of both. The analyses A and B and those under III form a parallel experiment conducted on a deproteinized solution and show the same effect. Finally reference to the analyses under II and IV shows that in an aqueous extract of cortex both chemical assays are as high as the physiological assay while the only apparent effect of aeration has been to oxidize the adrenaline side chain without destroying the catechol nucleus so that the molybdate assays IC and IIC are the same while the pressor assay of IIC is notably lower than IC. The results therefore lend no support to the hypothesis of "virtual" adrenaline.

Consideration of the chemical estimations shows that the persulphate figures vary too widely from the pressor assays to allow any useful analysis. On the other hand the molybdate estimations which in the cortical extracts are always in excess of the pressor assay show in the medullary extracts a mean difference of only 7%. This suggests the presence in the cortex of a catechol substance other than adrenaline. The discrepancies between physiological and colorimetric assays of suprarenal extracts noted by numerous investigators including, more recently, Frowein [1922] and Maiweg [1922], support this belief. Annau *et al.* [1932] postulated the existence in the medulla of an unstable derivative "nov-adrenaline", having many times the activity of free adrenaline. Euler however [1933], using a Pulfrich "Stufenphotometer" and a modified form of the Vulpian (iodine) reaction, concluded that in medullary extracts there is no significant difference between chemical and physiological assays. Less perfect agreement

was reported by Schild [1933] in a comparison of the iodine, persulphate (modification of Barker *et al.*) and Folin methods as applied to medullary extracts. In each case, the physiological exceeded the colorimetric assay and where comparison of the latter was possible best agreement was obtained in the order named. Schild has criticized the persulphate method adversely because of the somewhat haphazard development of colour which, even in pure adrenaline solutions, makes direct comparison impossible, although reasonable results can be obtained by taking the maximum "red" reading in a tintometer. The analyses reported in Tables I and II were carried out by matching in a colorimeter against a standard solution made by weighing out pure adrenaline and dissolving it in slightly more than 1 equiv. of dilute H_2SO_4 . A standard time at room temperature of $2\frac{1}{2}$ hours was found to be sufficient for development of the colour which is stable for many hours in the absence of air.

Examination of various inhibitory factors which might operate suggested either incomplete removal of protein by the method adopted or variable temperature effects. An extract of whole gland was therefore made with dilute H_2SO_4 , and the precipitation of protein completed at pH 5.5 by adding a little trichloroacetic acid to the hot solution. Persulphate estimation at various temperatures up to 40° was in each case wholly unsatisfactory as compared with the iodine method of Schild [1933] who obtained similar results for both methods of deproteinization. The only effect of elevated temperature was to strengthen the yellow component of the colour. The persulphate method therefore cannot be recommended except when used with a tintometer, nor can the analyses by this method reported in Exps. 1 and 2 be regarded as satisfactory.

It was thought that a final commentary both on the value of this method and on the existence of "virtual" adrenaline should be made by carrying out a more strict comparison using the adrenal glands of the horse. Quantities of 11 g. minced cortex and 4 g. medulla were therefore treated as follows for 3 hours:

- I. Extracted with $N/20 \text{ H}_2\text{SO}_4$.
- II. Extracted with H_2O .
- III. Aerated in 1% Na_2CO_3 .
- IV. Aliquot of cortical extract from I aerated in alkali.

The results are given in Table III (mg./10 g. tissue).

Table III.

Sample	Persulphate	Iodine		Molybdate	Pressor assay
		4.9	5.2		
I. C	2.7	85	85	—	6.8
II. M	32.5	4.2	4.4	93	95
C	5.5	—	—	—	4.9
III. M	0	0	0	—	10.3
C	2.6	—	3.0	—	3.6
IV. C*	—	—	0	—	0.8

* Referred to the whole of CI as "aliquot".

In these solutions 6 hours at $22-24^\circ$ were required for maximum colour development by persulphate. The estimations by iodine were duplicated so that the effect of varying certain conditions such as reaction time, excess of reagent and adrenaline concentration could be gauged. The high ascorbic acid content made application of the molybdate reaction to most of the solutions impossible. The analyses, presented in Table III, bear out the previous failure to reproduce the experiments on which the concept of "virtual" adrenaline is founded, for which at present no satisfactory explanation can be advanced.

Distribution of catechol substances in the suprarenal glands of the bullock.

As the discrepancy between physiological and chemical estimations of adrenal extracts is generally attributed to the latter it must be stated that the iodine method gives readily reproducible results when carried out under the conditions stated by Schild and that the colour developed is constant over wide variations in the amount of reagent added. The figures presented so far suggest that one source of error not previously examined may lie in the distribution in the gland of catechol substances other than adrenaline, i.e. in the difference between the iodine and molybdate assays as shown in Table IV. The first extract of each pair was made with $N/20$ H_2SO_4 and the second with 5% trichloroacetic acid.

Table IV. *Comparison of assays for medulla and cortex (mg./g. tissue).*

	Adrenaline	Total "catechol"	Pressor assay
Medulla	11.0	12.6	—
	11.3	13.3	12.1
Cortex	0.77	1.24	—
	0.71	1.15	0.65

The cortex apparently contains about 40% of its catechol material in a form which has little or no pressor activity. The proportion is much less in the medulla but is too great to be accounted for by possible admixture with cortical tissue in dissection. The following factors which might influence the molybdate estimation were further investigated:

Effect of pH. 4 ml. of medulla extract suitably diluted at pH 5.5 were treated with 5 ml. acetate buffer and 4 ml. reagent. The mean variation in colour intensity was shown to be only about 2% over the pH range 3.6–5.6 if the standard be developed at the same value.

In the cortical extract the effect seemed to lie in the opposite direction, owing possibly to the much greater interference of ascorbic acid which can be minimized by selecting a fairly high pH but one at which adrenaline itself is quite stable—say 5.4.

Effect of ascorbic acid. A test made as quickly as possible before further change showed that the yellow colour given by ascorbic acid is about one fortieth as effective as that of an equal weight of adrenaline and in fresh extracts would lead to a maximum error of about 6% in the cortex and 0.3% in the medulla. Other more sensitive reagents, such as Folin's, which are reduced by ascorbic acid give rise to more serious error. The quantitative separation of adrenaline and ascorbic acid cannot readily be carried out for routine analysis. Lead acetate has been used to remove the vitamin but there is disagreement as to the effective pH [Zilva, 1927; Smith & King, 1931; Emmerie, 1934]. 100 ml. cortex extract containing 50 mg. ascorbic acid were therefore treated with 20 ml. 10% lead acetate and adjusted with ammonia to pH 6.0, 7.2 and above 10.0. The procedure was unsatisfactory as each of the three precipitates was found to contain both "catechol" and ascorbic acid. A simpler method is to destroy the latter by aeration. According to Ahmad [1935], with ordinary exposure of such extracts oxidation commences about 3 hours after preparation. The effect on solutions kept sealed at 0° is shown in Table V (Exp. 1) by the increase in titre against a constant volume of indophenol solution.

Exp. 2 gives the analyses made 3 weeks after extraction of samples from another batch of glands. The yields are less but lie within normal variation

Table V. *Rate of oxidation of ascorbic acid at 0°.*

Exp.	Time in days	Titre (ml.)		M (mg./g.)		C mg./g.	
		M	C	Adrenaline	"Catechol"	Adrenaline	"Catechol"
I	0	4.3	2.3	—	—	—	—
	1.5	5.3	4.0	11.8	11.8	0.66	—
	6.5	14.3	11.3	—	—	—	1.08
II	—	—	—	9.1	10.2	0.41	0.74
	—	—	—	9.4	10.2	0.41	0.84
	—	—	—	8.9	9.9	0.42	0.82

[Schild, 1933]. In both groups the ratio of total "catechol" to adrenaline remains almost constant at 1.1 (medulla) and 1.7 (cortex) respectively. This suggests that the bio-synthesis of adrenaline may be regarded as a two-stage process in which an intermediate catechol substance is first elaborated by the cortex and is then carried into the medulla and converted into adrenaline. Further work along these lines is being carried out.

SUMMARY.

1. Oxidations have been carried out in alkaline media with the cortical and medullary tissues of the suprarenal glands of the horse and bullock. Chemical and physiological assays of the resulting solutions do not support the hypothesis of the existence in the adrenal gland of "virtual" adrenaline.

2. The distribution of catechol substances in the gland suggests that the cortex may play some part in the elaboration of adrenaline.

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