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A POSSIBLE MECHANISM OF ADRENALINE STABILIZATION IN THE ADRENAL GLAND

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It has been amply demonstrated that acetylcholine, before its release from nerve endings, exists in the form of a pharmacologically inactive, non-dialysable and denaturable protein complex (Loewi, Hagen, Kohn, and Singer, 1937; Kahane and Levy, 1937; Mann, Tennebaum, and Quastel, 1938, 1939; Brown, 1937). It also seems likely that "sympathin," before liberation from adrenergic nerve endings, must exist there in some stable stored form (Rosenblueth, 1937). In the isolation of adrenaline from the adrenal medulla, Kendall (1932)—in a note not subsequently expanded—reported the occurrence in the gland of lactyladrenaline, stable to hydrolysis, which he suggested might be an intermediate in the formation of adrenaline. The presence of lactyladrenaline and lactylnoradrenaline in hydrolysates of adrenal medullary tumour tissue has been well demonstrated by the careful investigations of Crawford (1951). The compounds were characterized by migration on paper and were compared with synthetic lactyladrenaline and lactylnoradrenaline. He was able to show that these compounds did not exist to any detectable extent in glands which had not been treated with strong acid during the preparation of the extracts, and concluded that they were artifarcts formed in the extraction procedure. Szent-Györgi and Svirbely (1932) also reported that extracts of the adrenal medulla contained a substance which they suggested was an ester of adrenaline.

Utevsky and Osinskaya (1938) and Utevsky and Levantzeva (1938) have suggested that adrenaline in the adrenal gland may be combined with protein. Evidence will be presented in this paper which, it is believed, lends support to this hypothesis in that there appears to be a protein material present in the adrenal medulla which is capable of stabilizing adrenaline and dihydroxyphenylalanine against atmospheric oxidation.

METHODS

Adrenal glands were obtained from two sources—fresh glands from local abattoirs, and frozen glands donated by the Armour Research Laboratories. The frozen material was prepared by dropping fresh adrenal glands on to dry ice. They were subsequently kept in a deep-freeze cabinet at −20° C., and were not allowed to thaw before use.

After dissecting away the cortex of the gland, homogenates were prepared either by the use of a Waring Blendor, or by grinding the medulla at 4° C. with washed quartz sand in a mortar. The ground material was diluted to a final concentration of 100 mg./ml. with either M/40-phosphate buffer or Krebs-Ringer soln., centrifuged at 800 *G* for 20 to 30 min. at 4° C. to remove cell fragments, and stored in a refrigerator until used. This period varied from 0.5 to 1.5 hr.

It had previously been found that the tissue extract described above would greatly retard the autoxidation of added dihydroxyphenylalanine (DOPA) as well as adrenaline in phosphate buffer at *p*H 7.8 (see Table I and Fig. 1). The effect of the "stabilizer" was therefore measured by comparing the time that elapsed before autoxidation began in solutions of DOPA at *p*H 7.8, and the time required for oxidation of the same amount of DOPA to start in the presence of various amounts of the stabilizer-containing mixture at the same *p*H.

In order to prevent the autoxidation of DOPA before the beginning of the measurement period the substance was dissolved in M/40-monobasic potassium phosphate, which poised the *p*H of the solution at about 5.0, and effectively prevented autoxidation. 0.5 ml. of this mixture and either 0.5 ml. of the monobasic phosphate, or of the solution to be tested, suspended in monobasic phosphate, were placed in the main vessel of the Warburg manometer. When measurements of autoxidation were desired, 1.0 ml. of M/2-dibasic sodium phosphate was added from the side arm, bringing the concentration of phosphate buffer to M/4 and a ratio of secondary to primary phosphate of 20:1, which gave a *p*H of about 7.8. The actual *p*H was slightly lower, owing to the action of the carboxyl group on the DOPA. There was no

TABLE I

THE EFFECT OF VARIOUSLY TREATED AQUEOUS EXTRACTS OF THE ADRENAL MEDULLA ON THE AUTOXIDATION OF DOPA AND ADRENALINE AT *p*H 7·8

All main vessels contained 0·5 ml. of 120 mg.% DOPA in M/40-KH_2PO_4 at *p*H 5·1. All sidearms contained 1·0 ml. M/2-Na_2HPO_4 at *p*H 8·2. Resulting *p*H on mixing, 7·8.

No. of Expt.	Added to DOPA in Main Vessel	Added from Sidearm at Mixing	Age of Extract (Hr.)	Length of Lag Period (Min.)	Pre-mix Q_{O_2} (Wet Wt.)	Post-mix	
						Lag Period Q_{O_2} (Wet Wt.)	Autoxidation Q_{O_2} (Wet Wt.)
1	M/40-KH_2PO_4	M/2-Na_2HPO_4		0 0	0·16 0·00	— —	1·13 1·11
	Centrifuged extract	M/2-Na_2HPO_4	24	60* 45* 30*	0·22 0·08 0·12	0·18 0·26 0·4	— — —
	Uncentrifuged extract			15 0 0	0·12 0·1 0·13	— — —	0·50 0·46 0·48
2	M/40-KH_2PO_4	M/2-Na_2HPO_4		0 0	0·0 0·0	— —	1·78 1·71
	Centrifuged extract	M/2-Na_2HPO_4	96	15 15	0·5 0·0	0·20 0·32	1·32 1·22
	Dialysed extract	M/2-Na_2HPO_4	96	0 0	0·0 0·0	— —	1·39 1·31
3	M/40-KH_2PO_4	M/2-Na_2HPO_4		0 0	0·12 0·14	— —	1·61 1·84
	M/40-KH_2PO_4	Copper 1×10^{-3}M	24	0 0	0·10 0·08	— —	1·74 2·04
	Centrifuged extract	M/2-Na_2HPO_4	24	30 30	0·14 0·10	0·26 0·32	2·01 2·40
	Centrifuged extract	Copper 1×10^{-3}M	24	0 0	0·1 0·08	— —	2·42 2·91
	Uncentrifuged extract	M/2-Na_2HPO_4	24	75* 30	0·20 0·09	0·50 0·5	— 1·31
	Uncentrifuged extract	Copper 1×10^{-3}M	24	0 0	0·14 0·13	— —	1·66 2·31
	Dialysed extract	M/2-Na_2HPO_4	24	75 45	0·12 0·09	0·12 0·05	0·32 0·45
4	M/40-KH_2PO_4	M/2-Na_2HPO_4	24	0 0	0·0 0·0	— —	1·47 1·45
	Centrifuged extract	M/2-Na_2HPO_4	24	30 30	0·04 0·1	0·28 0·30	1·62 1·42
	Uncentrifuged extract	M/2-Na_2HPO_4	24	45 60	0·1 0·09	0·32 0·35	0·92 1·68
	Uncentrifuged extract	$CuSO_4$ 1×10^{-3}	24	0	0·5	—	2·54
	Dialysed extract	M/2-Na_2HPO_4	24	60* 90*	0·0 0·1	0·19 0·24	— —
	Dialysed extract	$CuSO_4$ 1×10^{-2}M	24	0 0	0·19 0·24	— —	0·83 0·76
5	Centrifuged extract	M/2-Na_2HPO_4	2	105* 75* 60* 105* 75* 45	0·05 0·08 0·07 0·0 0·0 0·06	0·19 0·20 0·15 0·06 0·06 0·06	— — — — — 0·22
	Centrifuged extract	$CuSO_4$ 1×10^{-2}M		0 0 0 0 0 0	0·02 0·08 0·16 0·08 0·03 0·06	— — — — — —	1·13 1·36 1·33 1·14 1·20 1·30
6	(Added to adrenaline in main vessel) M/40-KH_2PO_4	M/2-Na_2HPO_4	1	30 30	0·0 0·0	0·0 0·0	0·24 0·24
	Centrifuged extract	M/2-Na_2HPO_4	1	180* 150*	0·01 0·01	0·02 0·02	— —

* Experiment terminated.

measurable difference between the *p*H of the DOPA solution alone and that of the DOPA homogenate when both were treated in this way. Accordingly, any variations in the rate of DOPA autoxidation are not felt to be due to differences in *p*H.

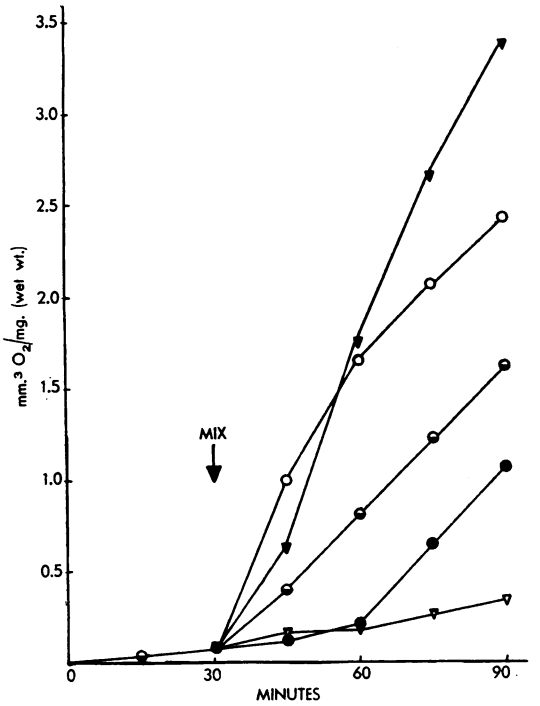

FIG. 1.—Oxidation of DOPA in the presence of variously treated extracts showing the presence of the tissue inhibitor. ◐——◑ DOPA autoxidation (*p*H 7.8). ▼——▼ Boiled extract of medulla. ○——○ Extract of medulla plus $CuSO_4$ (1×10^{-3}M). ●——● Extract of medulla. ▽——▽ Dialysed extract of medulla.

It should be emphasized that, under the conditions described above, the result of the addition of secondary phosphate to DOPA in primary phosphate is an immediate uptake of oxygen, and, in a very short time, a darkening of the solution by oxidation products. Thus in the test vessels, if no substance in the extract is capable of stabilizing DOPA against oxidation, the oxygen consumption of the test parallels that of the control. However, in an experiment so arranged, the presence of a stabilizing substance can be detected by a lack of oxygen consumption after the addition of secondary phosphate.

This test was chosen to avoid the necessity of depending upon the presence of adrenaline in the extract. Some of the fractions examined were free of adrenaline, and the addition of DOPA ensured the presence of an autoxidizable substance. DOPA was chosen rather than adrenaline because it was available in a pure state. The experiments have also been run with adrenaline and the results are in complete agreement.

RESULTS

Table I shows the effect of various treatments on the capacity of homogenates of the adrenal medulla to delay the autoxidation of added adrenaline and DOPA. All fractions were prepared in M/40-monophosphate at *p*H 5.0. It can be seen from Table I that the presence of the tissue extract is capable of retarding the autoxidation of DOPA and adrenaline for a considerable time, depending upon the age of the extract and the treatment it has received.

Table I also shows that the amount of oxygen consumed by the boiled extract in the presence of DOPA greatly exceeded the autoxidation of DOPA, and also the oxygen consumed by the unboiled material after the addition of DOPA. It seems likely that the extra oxygen consumption reflected the oxidation of liberated naturally occurring autoxidizable substances in the gland, which presumably were combined in the fresh gland in a stable complex.

The age of the tissue extract appears to have an effect on the activity of the "stabilizer." In Table I, Expts. 1 and 2 were performed on the same tissue extract, except that before Expt. 2 it had been kept for three days in the refrigerator in M/40 monophosphate buffer. When the extract was tested a second time the lag period was significantly reduced. The reduction in "stabilizing" efficiency was actually greater than would appear from the table, since the one-day-old homogenate had permitted no oxidative activity at the time the experiment was ended. The "ageing" effect was accelerated by increasing the temperature at which the homogenate was kept.

Copper, cobalt, and manganese were tested at a concentration of 1×10^{-3}M in order to determine what effect heavy metals might have on the system under consideration. The addition of copper abolished the lag period completely—as did cobalt and manganese—and increased the oxygen consumption markedly.

Dialysis of the tissue extract against distilled water enhanced the "stabilizing" capacity of the tissue extract. The dialysate, when tested, showed no "stabilizer" activity and, in fact, it autoxidized spontaneously when the *p*H was raised to 7.8. This observation suggested that the augmented action of the dialysed extract was due to the removal of naturally occurring materials from the complex, which made a greater amount of the stabilizer available for combination with the added DOPA.

The effects of boiling, and of dialysis, upon the tissue extract suggested that the retarding of oxi-

dation might be associated with the protein fraction of the extract. Accordingly, three protein precipitants were tested for their effect on the stability of DOPA in the presence of homogenates, as shown in Table II. Table II indicates clearly

TABLE II

DECREASE IN DOPA-STABILIZING CAPACITY OF ADRENAL MEDULLA EXTRACTS AFTER TREATMENT WITH VARIOUS PROTEIN PRECIPITANTS

All main vessels contained 0.5 ml. of 120 mg.% DOPA in M/40-KH_2PO_4 at *p*H 5.1, and 0.5 ml. of soln. to be tested in monophosphate buffer. All sidearms contained 1.0 ml. M/2-Na_2HPO_4 at *p*H 8.2. *p*H after mixing 7.8.

Solution Tested	Test			Length of Lag Period (Min.)	Pre/Post-mix mm.^{3} O_2/mg./hr.
	Millon	Biuret	Ninhydrin		
Phosphate buffer ..	++	++	++	0	0/0·9
Extract				90*	0/0
Extract after treatment with:					
5% trichloroacetic acid	−	−	−	30	0/0·76
Zinc sulphate } Barium hydroxide }	−	−	−	30	0/0·76
2% phosphotungstic acid	±	±	±	60	0/0·13

++ = strong positive. ± = weak positive. − = negative.
* Experiment terminated after 90 min.

that the presence of proteins in the extracts correlates completely with the stabilization phenomenon. For example, with 5.0% trichloroacetic acid and the zinc-barium reagents—and where the extracts were negative to the Millon, biuret and ninhydrin tests—the resulting protein-free solution did not delay the autoxidation of DOPA beyond the first reading period. However, with phosphotungstic acid-treated solutions—where the tests for proteins were all weakly positive—some retarding effect on the autoxidation of DOPA and adrenaline was observed. In the untreated extract no autoxidation could be detected even at the end of the experiment, whereas the controls, containing DOPA, but no extract, at the same *p*H, showed no lag period; and autoxidation proceeded immediately after the addition of secondary phosphate. From these results it seems necessary to conclude that there is a correlation between the extent to which proteins are removed from the adrenal homogenate and the rapidity with which DOPA is oxidized in the solution.

Since it had been suggested that some protection against autoxidation of adrenaline might be furnished by various sulphydryl compounds, or by ascorbic acid, determinations were carried out to assess the role of these compounds in the stabilization of the hormone in gland extracts.

Experiments performed in the presence of M/1000-sodium iodoacetate, an effective inhibitor of sulphydryl-group action, showed no decrease in the time elapsed before autoxidation occurred. In the ammonium sulphate precipitates, the nitroprusside test for sulphydryl compounds proved negative. Accordingly, it is felt that compounds of this type cannot account for the observed stabilization.

Analyses, using the 2:4-dinitrophenol method, detected little or no ascorbic acid, either before or after autoxidation proceeded. Therefore, this compound could not have accounted for the observed stabilization.

Furthermore, stronger evidence than the foregoing may be found upon consideration of the results obtained with 6% trichloroacetic acid. This precipitant removes proteins, most proteoses and peptones, and even some amino acids, but leaves behind sugars and ascorbic acid. One would expect, therefore, if ascorbic acid were instrumental in the stabilizing effect, that the trichloroacetic acid fractionation would result in enhanced stabilizer activity in the protein-free fraction. This did not occur; ascorbic acid may accordingly be eliminated as a cause of the adrenaline stabilization reported in this paper.

Fractionation procedures are being carried out in an attempt to show that it is possible to obtain a purified protein, which, when introduced into solutions containing DOPA or adrenaline, will retard the oxidation of these substances. The addition of saturated ammonium sulphate solution, to a final concentration of 55% saturation, resulted in the sedimentation of a large proportion of the "stabilizer" activity. Further purification of this fraction is being carried out.

DISCUSSION

The remarkable stability of adrenaline against autoxidation in extracts of the adrenal medulla suggests that adrenaline does not exist in the gland in the same chemical state as after chemical extraction, as pointed out by Kendall (1932) and by Rosenblueth (1937).

Having eliminated the possibility that the redox properties of ascorbic acid, and of compounds containing free sulphydryl groups, are responsible for the observed stability, several alternative explanations may account for the differences in chemical properties between the adrenaline in the gland and the isolated product. Kendall (1932) isolated lactyladrenaline from the adrenal medulla. Crawford (1951), however, cast considerable doubt on the possibility that lactyladrenaline represents the actual storage form of the hormone, but sug-

gested that it is formed as a result of the extraction procedure employed.

Another possibility is that adrenaline does not exist in the gland at all, but that the vasopressor substance is enzymatically produced and immediately liberated from the gland, upon appropriate stimulation. This supposition has a logical weakness which, while not fatal, argues against it. Standard methods for the extraction of adrenaline usually begin with a treatment of the minced gland with 95% ethanol, or some other equally efficient protein precipitant. After a short time such treatment would render normal enzymatic changes most unlikely, and one would expect that the extraction procedure would yield large quantities of an inactive precursor, but little or no formed adrenaline. No such precursor has been identified in these extracts, and large amounts of adrenaline have been obtained.

A third possibility, which would account for both the chemical differences between synthetic adrenaline and the glandular vasopressor substance, is that the vasopressor principle is combined with a protein in the gland in such a manner that, upon demand, adrenaline may be released into the solution. Such a proposal would require as a prerequisite a protein capable of combining with adrenaline, and one which would, in this combination, form a substance which was non-diffusible and non-oxidizable under living conditions. This protein would also have to be related to the physiological activity of the gland in such a manner that stimulation of the gland would result in the destruction of the adrenaline-protein complex, and liberation of the hormone into the circulation.

The evidence presented in this paper indicates that there exists in the adrenal gland a protein which can prevent the autoxidation of adrenaline and DOPA. Although our experiments were not designed to localize adrenaline on particulate cellular components, our data are entirely consistent with the observations of Blaschko and Welch (1953), who showed that adrenaline is associated with the protein-rich mitochondrial fraction of the cells of the adrenal medulla.

Summary

1. The metabolism of the adrenal medulla has been studied on extracts in the presence of various substrates.

2. Protein, capable of preventing the autoxidation of dihydroxyphenylalanine at *p*H 7.8, exists in the adrenal medulla.

3. It is suggested that this protein acts as a " stabilizer " by forming a complex with adrenaline in the gland and so preventing the autoxidation of adrenaline under physiological conditions.

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