Brit. J. Pharmacol. (1951), **6**, 318.

ENZYMES AND THEIR SUBSTRATES IN THE ADRENAL GLAND OF THE OX

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(Received March 19, 1951)

Since the formation and the release of pressor agents are the chief functions of the adrenal medulla, it seemed of interest to investigate the activities of enzymes which may be connected with these two functions in the adrenal gland itself.

Ox adrenal glands were used throughout because the medulla and the cortex can be separated fairly easily, the cortex being but little folded and the medulla being very thick and containing few inclusions of cortical tissue. The proportion of cortex to medulla is small, being of the order of about 1.5 to 1.

Since the innervation of the medulla is cholinergic, cholinesterases were determined. The medulla is known to contain a fairly active enzyme hydrolysing acetylcholine, whereas the cortex shows little activity (Antopol and Glick, 1940; Langemann, 1942). Amine oxidase activity and histaminase ("diamine oxidase") activity were also measured, since these enzymes may influence the production and degradation of aromatic pressor amines and their release into the blood stream by histamine. The presence of amine oxidase in sheep adrenal glands has previously been described by Bhagvat, Blaschko, and Richter (1939), and in the medulla and cortex of ox by Schapira (1945). The former authors made no attempt to separate the medulla from cortex, and the latter author used only a qualitative method. Extracts from mammalian kidney (Holtz, Heise, and Lüdtke, 1938) and liver (Blaschko, 1939) are able to form pressor amines like hydroxytyramine (Holtz *et al.*, 1938) and *nor*adrenaline (Blaschko, Burn, and Langemann, 1950), and it was therefore of interest to find out if similar processes occurred in the adrenal gland. Schapira (1946), working with extracts of whole adrenal glands of the guinea-pig, was not able to demonstrate the presence of DOPA-decarboxylase.

METHODS

The easiest and quickest way of separating medulla from cortex was by freezing the gland at $-15°$ C. after its removal from the ox, and separating the darker coloured cortex from the lighter medulla by means of a scalpel. To test the relative completeness of the separation, extracts of the cortex and of the medulla were made and tested on the arterial blood pressure of a spinal cat. One such medullary extract was found to give a blood pressure rise equivalent to 12.5 mg. adrenaline per g. of fresh tissue, whereas the cortical tissue extract had only about 1 per cent of the pressor activity of the medullary extract.

* With a grant of the Swiss "Stiftung für Biologisch-Medizinische Stipendien."

One of the peculiar features of adrenal medullary tissue is the enormous content of adrenaline and *nor*adrenaline, which amounts to about 10 per cent of its dry weight. Figures are given in Table IV also for the relative percentages of the two main pressor agents, adrenaline and *nor*adrenaline, determined by the method of Burn, Hutcheon, and Parker (1950).

EXPERIMENTS

Cholinesterases.—The tissues were ground in a mortar with sand or silica and then suspended in Krebs's bicarbonate-Ringer. The cholinesterase activity was determined manometrically at 37.5° C. in a gas mixture of 95 per cent N_2 and 5 per cent CO_2, 2.0 ml. of tissue suspension, containing either 200 mg. wet weight medulla or 400 mg. wet weight of cortex, being used. The side bulb contained 0.3 ml. of substrate. The final concentrations used were: acetylcholine bromide 0.026 M, acetyl-β-methylcholine chloride 0.039 M, and benzoylcholine chloride 0.0078 M. The initial reading was taken 3 or 5 min. after tipping, and subsequently at 3 or 5 min. intervals for the following 30 minutes.

The figures for q_{CO_2} given in Table I represent the μl. CO_2 liberated by 1 g. (wet weight) of tissue per 60 min. These values were calculated from 3–15 min. or 5–15 min. after tipping, i.e., when the enzymic hydrolysis was proceeding at a linear rate. The readings were corrected for spontaneous hydrolysis of the substrates and for the extract blanks, which were small.

Addition of 0.0023 M-*l*-adrenaline or 0.0046 M-*dl-nor*adrenaline to a very active preparation of dog's caudate nucleus resulted in an 11 per cent inhibition of the hydrolysis of acetylcholine.

TABLE I

CHOLINESTERASE ACTIVITY OF ADRENAL TISSUE EXTRACTS

Preparation	q_{CO_2} values (μl. CO_2/g. wet tissue/60 min.) for different substrates		
	Acetylcholine	Acetyl-β-methylcholine	Benzoylcholine
Medulla:			
Exp. 1	1,950	590	50
,, 2	2,650	1,470	0
,, 3	2,020	1,050	0
Cortex:			
Exp. 1	230	120	35
,, 2	115	15	0
,, 3	290	90	0

Amine oxidase.—The tissues were ground with sand in a mortar and suspended in M/15 phosphate buffer, $pH = 7.4$. Some preparations were dialysed against phosphate buffer for 3 or 22 hours at 4° C.; this procedure does not affect the amine oxidase activity. The enzymic activity was measured manometrically at 37.5° C. in O_2. Into 1.6 ml. of tissue suspension, containing 400 mg. (wet weight) of tissue, 0.4 ml. of 0.05 M-tyramine hydrochloride or -*iso*amylamine hydrochloride was tipped and readings were taken during the subsequent 30 to 60 min. The CO_2 was absorbed by 0.3 ml. N-NaOH in the centre well of the flask.

The figures given in Table II are expressed as q_{O_2}, i.e., the μl. O_2 consumed by 1 g. (wet weight) of tissue per 60 min., for the blanks and for the experimental flasks. The q_{O_2} was calculated from the initial rate of oxidation, while the reaction was approximately linear. The blanks were often high, especially in cortex, but in this tissue they were reduced almost to zero by dialysis. In medullary suspensions dialysis was less effective in reducing the blanks. This may be due to oxidation of

TABLE II

AMINE OXIDASE ACTIVITY IN OX TISSUE EXTRACTS

Oxygen uptake of tissue suspensions without and in presence of substrate; $q_{O_2} = \mu l.O_2/g.$ tissue/60 min. In the last two columns blank oxygen consumption has been deducted

Preparation	Exp. No.	q_{O_2} blank	q_{O_2} Tyramine	q_{O_2} *Iso*amylamine
Medulla	1	247	418	—
	2	97	140	—
	3	237	225	50
	4	225	262	141
	5*	160	530	150
	6	260	350	150
	7*	180	590	160
Cortex	1	96	—	64
	2	168	274	—
	3	350	125	(19)
	4	330	250	90
	5*	20	730	70
	6	660	300	200
	6a†	190	380	60
	7*	40	640	100
Kidney	1	125	3,180	1,370
	2*	30	3,180	1,620
Liver	1	180	3,030	1,380
	2*	180	4,140	1,770

* Dialysed extracts (22 hours). † Dialysed extracts (3 hours).

aromatic amines (adrenaline and *nor*adrenaline) which had not been removed completely by dialysis. For comparison, figures for the amine oxidase activity in similarly prepared suspensions from liver and kidney of the same species have been included in Table II; these figures show that the activity of the adrenal tissue is much lower than that of liver or kidney.

Histaminase ("diamine oxidase").—These experiments were carried out in the same way as those for the measurement of amine oxidase, with the difference that cadaverine (pentamethylene diamine) dihydrochloride was used as a possible substrate and that the extract was centrifuged for 5 min. at 2,000 rev. per min. in order to remove particulate matter. The supernatant was used in the manometric experiments.

As in the experiments on amine oxidase, the oxygen uptake of the blanks was high, but there was no excess uptake of oxygen in the presence of cadaverine. Histaminase seems to be absent from these tissues or, at least, only present in amounts too small to be measured by the method employed.

DOPA-decarboxylase.—The method used was that described by Blaschko (1942); the tissue was ground with sand in a mortar, suspended in an equal amount of M/15 phosphate buffer, *p*H = 7.4, and centrifuged. The rate of decarboxylation was followed manometrically at 37.5° C. under nitrogen atmosphere. To 1.6 ml. of the supernatant, corresponding to 800 mg. of fresh tissue, 0.4 ml of a 0.01 M solution of L-DOPA was tipped from the side bulb of a conical manometer flask at the beginning of the experiment. Readings were taken 3 or 5 min. after tipping and then at short intervals, usually up to 30–60 min. until the reaction had come to a standstill. In order to determine the CO_2 retention in the suspension, 0.3 ml. N-HCl from the centre well of the flask was then mixed with the contents. In some experiments semicarbazide was added to give a concentration of 0.01 M, in order to inhibit the enzyme reaction.

The figures for q_{CO_2} given in Table III represent the μl. CO_2 formed by 1 mg. (wet weight) tissue in 60 min. The blanks were usually low. In most of these experiments the q_{CO_2} values were calculated from the first 3 or 5 min. readings after tipping. This was necessary, as the initial rate of the reaction was not maintained for long. Although the initial activity was rather high and comparable to that of guinea-pig kidney, the reaction appeared to come to a standstill before all

TABLE III

DECARBOXYLATION OF DOPA BY OX ADRENAL TISSUE

The figures give the q_{CO_2} per mg. of tissue per 60 min. The values for the blanks, which were small, have been subtracted from these figures

Medulla	The following results were obtained under the experimental conditions described in the text. The q_{CO_2} were: Experiment 1 .. .. .. 0.775 2 .. .. .. 0.800 3 .. .. .. 0.900 4 .. .. .. 0.825 5 .. .. .. 0.825 Mean result from 20 experiments, including some in which amount of tissue, amount of substrate, *p*H, and buffer were slightly modified: q_{CO_2} = 0.695 (lowest figure, 0.226; highest, 1.150)
Cortex	Mean result from 10 experiments: q_{CO_2} = 0.077 (lowest, 0; highest, 0.250)
Kidney (same species) ..	q_{CO_2} = 0.250
Liver (same species) ..	q_{CO_2} = 0.250

the substrate had been decarboxylated. Fifteen experiments were carried out with the cortical extracts and thirty with extracts of medulla. These experiments include many in which the experimental conditions were slightly modified from those described above. Tissue concentration and *p*H were varied, but no higher rates of decarboxylation were obtained. A summary of these experiments is given in Table III.

Since it is known that DOPA-decarboxylase is a pyridoxal phosphate protein, it seemed likely that the enzyme might be inhibited by carbonyl reagents. This

was demonstrated in experiments with extracts from ox liver and kidney. In the presence of 0.01 M-semicarbazide, the DOPA-decarboxylase activity was inhibited by about 90 per cent. The CO_2 formation from DOPA in the presence of an extract of suprarenal medulla was similarly reduced.

Discussion

The experiments on cholinesterase activity of the adrenal gland are of interest, as they show that most of the high activity is localized in the medulla, the cortex containing only about a tenth of that activity. The enzyme is of the type which Mendel, Mundell, and Rudney (1943) described as " true " cholinesterase and Augustinsson and Nachmansohn (1949) as " acetylcholine esterase." This is in agreement with the observation by Ord and Thompson (1950) on the rat suprarenal, but these authors did not separate the medulla and the cortex, whereas the earlier investigators, who separated these tissues, did not at that time differentiate the two types of enzyme (Antopol and Glick, 1940 ; Langemann, 1942). In the ruminant, " pseudo " or " non-specific " cholinesterase is said to be absent (Gunter, 1946). This is confirmed by our observation on the adrenal gland of the ox, where the hydrolysis of benzoylcholine did not occur or was very small. Acetyl-β-methylcholine, however, was actively hydrolysed. The experiments reveal an interesting difference between the cholinesterase of adrenal tissues in the ox and those of the whale (Burn, Langemann, and Parker, 1951): in the latter species acetylcholine is hydrolysed rapidly, but acetyl-β-methylcholine only very slowly and benzoylcholine not at all.

The activity of amine oxidase in both medulla and cortex was found to be rather low in comparison with that in liver or kidney of the same species. Moreover, the small amount of tissue of the adrenal glands, compared with that of other organs with high amine oxidase activity, would mean that the adrenals contribute little to the total turnover rate for amines in the animal body. The experiments with dialysed extracts seem to indicate that the adrenal gland has a markedly higher activity towards tyramine as compared with *iso*amylamine. This may be considered as an adaptation of the enzyme to the type of amines usually present in the gland.

Since histamine is known to release pressor amines from the adrenal gland (Burn and Dale, 1926), the histaminase (diamine oxidase) activity was measured, but no activity of extracts of adrenal medulla or of cortex was found.

It may be of interest to put together (Table IV) the concentrations of the substrates of some of the enzymes here described which have been found to be present in medulla and cortex of the ox adrenal. It may be mentioned that choline oxidase activity was looked for, but not found in our experiments.

DOPA-decarboxylase activity in the adrenal medulla is particularly interesting. The activity is as high as that of the guinea-pig kidney, known to be the best source for this enzyme. The enzymic activity is about ten times higher in adrenal medulla than in cortex. It is also several times higher in the medulla than in the kidney or in the liver of the ox. This suggests strongly that DOPA-decarboxylase has an important function in the medullary tissue and that, in spite of the relatively small size of the adrenal gland, the enzymic activity in this tissue represents a significant portion of the total activity of this enzyme in the body of the ox.

Holtz *et al.* (1938) showed that the decarboxylation of DOPA by extracts of mammalian kidney resulted in the production of a pressor amine, hydroxytyramine.

TABLE IV

CONCENTRATIONS OF ENZYME SUBSTRATES IN OX ADRENAL TISSUE

Figures are given as μg. per g. wet tissue

Substrate	Gland No.	Medulla	Cortex
Adrenaline and *nor*adrenaline	1	12,500 (75%)*	140
	2	11,400 (50%)*	—
	3	16,500 (50%)*	—
Histamine (5 experiments) estimated by the method of Code (1937)		1.1–9.1 2.6 (mean value)	0.15–1.35 0.6 (mean value)
Acetylcholine†		0.6–0.72	0.12–0.2
Choline†		50	170

* The percentage means % adrenaline present together with *nor*adrenaline measured by the method of Burn, Hutcheon, and Parker (1950).

† These figures are taken from Feldberg and Schild (1934).

Hydroxytyramine has only about 1/35th of the pressor activity of adrenaline, which makes it difficult to demonstrate the formation of this amine in the presence of the large amounts of adrenaline and *nor*adrenaline found in these extracts.

The fact that the enzymic decarboxylation of DOPA by medullary tissue extracts did not reach completion in any experiment, as it usually does with kidney extracts, may be due to inhibition by the reaction product. Blaschko (1942) found that the addition of the reaction product, hydroxytyramine, in a molar concentration ten times that of the substrate DOPA inhibited the reaction by 71 per cent. These findings were extended by Polonovski, Schapira, and Gonnard (1946), who reported that equimolar amounts of *nor*adrenaline, as well as sympatol (synephrin) and tyramine, inhibited the DOPA-decarboxylase activity of guinea-pig kidney extracts by 61–65 per cent. The possibility cannot therefore be excluded that the adrenaline or *nor*adrenaline present in medullary extracts in very high concentration caused an inhibition of the DOPA-decarboxylase. It seems worth considering whether this inhibition may not act as a regulating mechanism in the living animal; after physiological stimulation, when the content of pressor amines in the gland is relatively low, this enzyme might be more active than at rest, when the gland is full of these substances.

It has been shown that DOPA can be rapidly decarboxylated by the adrenal medulla (and less actively by the cortex), giving rise, very probably, to hydroxytyramine. It is known that with guinea-pig kidney extracts dihydroxyphenylserine can also be decarboxylated, although less rapidly than DOPA, and that *nor*adrenaline is formed in this reaction (Blaschko, Burn, and Langemann, 1950). It seems likely that in the kidney one and the same enzyme is responsible for the decarboxylation of the two amino-acids. These experiments therefore raise the question whether *nor*adrenaline can similarly be formed by decarboxylation in the adrenal medulla of the ox.

The DOPA-decarboxylase determinations were made in a nitrogen atmosphere. This is necessary, as in an oxygen atmosphere the amine oxidase present in the

same tissue extract would further degrade amines formed by decarboxylation. The experiments by Bing (1941) showed that kidneys, perfused with DOPA under hypoxic conditions, form a pressor substance which is similar in its action to hydroxytyramine. Amine oxidase, which is present in kidney in high activity, requires a good oxygen supply, and therefore was unable to destroy the amines formed in these experiments. In experiments on the perfused adrenal gland of the dog, Bülbring, Burn, and Elío (1948) showed that oxygen lack resulted in an output of pressor amines (adrenaline and *nor*adrenaline were not determined separately) into the venous blood. Under these conditions, therefore, the activity of the amine oxidase present in the gland can be expected to be low.

The experiments on the enzyme activities found in the adrenal gland suggest the possibility that the formation and the release of the pressor amines depend on the interaction of amine oxidase and DOPA-decarboxylase in the adrenal medulla itself, regulated in their functional state by the oxygen supply of the blood.

SUMMARY

1. Extracts of ox adrenal medulla and cortex have been prepared and examined for their enzymic activities.

2. The cholinesterase activity of the medulla was high, that of the cortex about one-tenth of the medullary extracts ; the enzyme hydrolysed not only acetylcholine but also acetyl-β-methylcholine ; the hydrolysis of benzoylcholine was negligible.

3. The amine oxidase activities of the medullary and cortical extracts were of about the same order ; tyramine was oxidized more rapidly than *iso*amylamine. There was no evidence of the occurrence of histaminase (diamine oxidase).

4. L-DOPA was found to be decarboxylated at a high rate in extracts of medulla.

I wish to thank Professor J. H. Burn for the hospitality of his laboratory and Dr. H. Blaschko for his advice and interest during this work.

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