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THE INACTIVATION OF ADRENALINE BY PHENOLASES

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The oxidation of adrenaline by enzymes may occur on the side chain or on the ring structure of the molecule. Oxidation on the side chain is brought about by an enzyme found in tissue extracts [Blaschko, Richter & Schlossmann, 1937*a*, *b*]. This enzyme, which is identical with tyraminase [Hare, 1928], is now generally called amine oxidase. The analysis of its action was made possible by the observation that it was not inhibited by cyanide [Blaschko & Schlossmann, 1936*a*]. Other enzymic systems attack adrenaline because it is a catechol derivative and they are inhibited by cyanide. The experiments described in this paper concern the inactivation of adrenaline by these systems.

From the work of Warburg and Keilin and their collaborators it is known that the enzymic systems which oxidize polyphenols contain either copper or iron, and it is therefore possible to classify them accordingly.

Catalysts containing copper. The best studied enzyme of this type is the polyphenol oxidase of potatoes [Kubowitz, 1937, 1938] and of mushrooms [Keilin & Mann, 1938]. Other enzymes which come into this group but have not yet been proved to be copper compounds are the phenolase of the mealworm and of *Sepia officinalis*. Laccase is also an enzyme of this type.

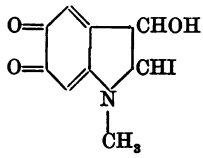
Catalysts containing iron. The most important representative of this class is cytochrome-cytochrome oxidase. It oxidizes polyphenols [Keilin & Hartree, 1936, 1938; Green & Richter, 1937], and is the chief respiratory system of all aerobic cells. The oxidation of adrenaline is probably more complex than in the case of polyphenol oxidase, because several stages of oxido-reduction are interposed between adrenaline and oxygen, namely the three cytochromes (*a*, *b*, *c*) and the oxidase. Another enzyme of this

group is peroxidase which is also a haematin compound [Kuhn, Hand & Florkin, 1931]. It occurs in plants and its chief source is horseradish.

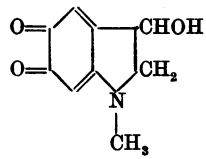
In the previous study on amine oxidase we were able to show that there exists a strict stoichiometrical relationship between inactivation of adrenaline and oxygen uptake. A similar analysis has not hitherto been made for the different cyanide-sensitive enzymes. This has been done in the experiments described in the present paper for polyphenol oxidase, cytochrome-cytochrome oxidase and peroxidase.

There was an additional interest attached to this analysis, since Bacq [1938] claimed that during the oxidation of adrenaline by phenolases a substance with inhibitory properties is formed. This substance he called adrenoxine. Bacq used various enzyme preparations derived from animal and plant tissues, and we have tried to repeat his observations with a similar preparation as well as with the purified enzymes at our disposal.

The oxidation of adrenaline catalysed by phenolases is similar to the oxidation of adrenaline by chemical agents, e.g. iodate, copper or iron salts. These reactions lead to coloured substances which have recently been identified [Richter & Blaschko, 1937; Green & Richter, 1937]. The probable formulae ascribed to the products of the oxidation by iodate (iodochrome of adrenaline) and of the closely related enzymic oxidation (adrenochrome) are given below. Adrenochrome corresponds in its



Iodochrome of adrenaline



Adrenochrome

structure to the red substance formed in the enzymic oxidation of tyrosine, the constitution of which was cleared up by Raper [1927] in his classical studies on tyrosinase.

Methods

Adrenaline solutions were incubated with enzyme preparations and the uptake of oxygen was measured manometrically. The initial adrenaline concentration was 1 in 1000. Usually 2 mg. adrenaline dissolved in the equivalent amount of hydrochloric acid in a total volume of 0·2 c.c. was placed in the side bulb of a conical manometer flask; the main flask contained the enzyme and 0·067 *M* sodium phosphate buffer *p*H 7·3 to bring the total volume to 1·8 c.c. The gas space of the manometers was filled with air, except in the experiments with peroxidase, where no

uptake of oxygen occurs and where the gas space was filled with nitrogen. The flasks were incubated in a bath at room temperature (17–20°). In a single experiment several manometers were set up with the same contents and the uptake of oxygen was measured over suitable intervals. From time to time one of the flasks was removed after a final reading, and the solutions tested at once for their pharmacological activity. It was usually unnecessary to take special precautions to protect the samples from further changes between the final reading and the biological assay, as the oxidation was already slow at the time of removal from the bath. In a few experiments with polyphenol oxidase where the assay was to be done whilst the rapid reaction was still in progress duplicate samples were set up. One was removed and the enzymic reaction was stopped by adding a drop of hydrochloric acid to the sample. At this moment the final reading was taken from the second sample.

In his experiments on the formation of adrenoxine Bacq has used adrenaline solutions 1 in 10^5, and our experiments concerned with the formation of adrenoxine were therefore done not only under the conditions just described but also with solutions (1 in 10^5), which were kept with the enzyme preparation at room temperature up to 24 hr.

The activity of the samples was assayed on the arterial blood pressure of decerebrate cats as previously described [Blaschko *et al.* 1937*a*], and in some cases of cats under chloralose anaesthesia. A few samples were tested on the isolated rabbit's intestine.

Enzyme preparations. The polyphenol oxidase was prepared according to Keilin & Mann [1938]; three preparations were used. One was made from *Psalliota*; it had, when tested on catechol, a Q_{O_2} of 40,000 (preparation A). The other two were from *Lactarius*; they differed in purity and activity, the Q_{O_2} of the more active and more purified preparation was 80,000 (preparation B), that of the less active 10,000 (preparation C). For the experiments on the formation of adrenoxine we used in addition press juice from unpeeled potatoes which was prepared on the day preceding the experiment. The cytochrome *c* was a 1 % solution obtained from horse heart according to Keilin & Hartree [1937]; the cytochrome oxidase came from pig's heart [Keilin & Hartree, 1938]. The peroxidase was a preparation from horseradish [Keilin & Mann, 1937]; its activity as measured by its purpurogallin number was 300. It was about 3000 times purified. We are indebted to Prof. D. Keilin, Dr E. F. Hartree and Dr T. Mann for the purified preparations used in this work.

Results

Polyphenol oxidase. Preparation A was used in two series of preliminary experiments in which the inactivation of adrenaline was determined without the uptake of oxygen. The enzyme was used in a 100 and 1000-fold dilution respectively. The initial concentration of adrenaline was 1 in 10^5. Addition of the enzyme to the adrenaline solution led almost instantaneously to the development of a red colour, the maximum redness being reached more quickly with the stronger enzyme solution. When the samples were tested 85 min. after the addition of the enzyme they had no action on the arterial blood pressure of the cat. When retested at different periods during the following 6½ hr. the samples remained inactive and did not acquire depressor activity. The colour of the solutions had by that time changed from red to yellow-brown and a slight precipitate had formed.

With preparations B and C inactivation and oxygen uptake were measured simultaneously. After the addition of adrenaline to the enzyme solutions an uptake of oxygen occurred which was complete in about 15 min. During this period the reaction was so rapid that the rate of oxidation was probably limited by the diffusion of oxygen into the solution, and our readings therefore give no true measure of the reaction rate. This phase was accompanied by the development of a red colour, the intensity of which increased until the rapid uptake of oxygen had ceased. If the oxygen consumption is expressed in atoms of oxygen per mol. adrenaline it will be seen that in the rapid reaction exactly 2 atoms of oxygen had been used up. After this period a very much slower uptake of oxygen continued for hours and eventually about 6 atoms of oxygen had been taken up. This is illustrated by the experiment of Fig. 1. The manometers were set up as follows:

Main flask: 1·7 c.c. phosphate buffer + 0·1 c.c. preparation B diluted 1 : 10.

Side bulb: 0·2 c.c. adrenaline hydrochloride (= 2 mg. base).

Inner tube: 0·3 c.c. *N* KOH.

$t = 19{\cdot}0°$; gas phase : air.

The second phase of oxygen uptake was again accompanied by a change in colour. The red colour faded slowly and was replaced by a yellow-brown colour. The assay of the samples on the cat's blood pressure showed that inactivation was complete when 2 atoms of oxygen had been taken up per mol. adrenaline. This is illustrated by Table I, which belongs

to the same experiment as Fig. 1. The amount of oxygen that should be consumed if the inactivation of adrenaline required 2 atoms per mol. is

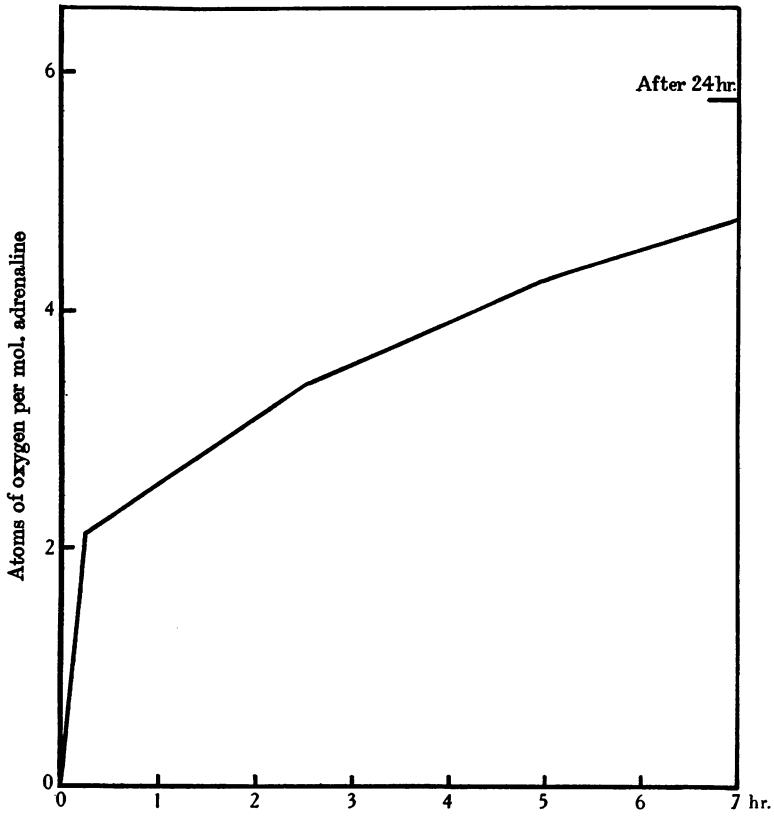


Fig. 1. Oxygen uptake of adrenaline with polyphenol oxidase.

TABLE I. Oxygen uptake and inactivation of adrenaline by polyphenol oxidase

Time of incubation in. min.	Oxygen consumed in c.mm. O_2	Activity expressed in % of initial activity	
		Calc.	Found
9	130	47	30
10	162	34	30
14·5	210	14	25
31	251	0	0·1
1521	710	0	0

245 c.mm. It can be seen from the data of columns 3 and 4 that inactivation was complete when this amount of oxygen had been taken up. The slight discrepancies between the expected and found values of activity

in the early determinations are of no significance; we have to consider that the oxygen uptake and the inactivation had to be measured in separate samples. When samples had lost their pressor activity and were retested at later stages of oxidation (up to 25 hr.) they never acquired a new pressor or a depressor action. We have carried out such experiments with solutions containing adrenaline in an initial concentration of 1 in 10^3 and 1 in 10^5 and with the enzyme preparations B and C. These results show that no substance like Bacq's adrenoxine is formed during the oxidation by relatively highly purified preparations of polyphenol oxidase. There was the possibility that the formation of adrenoxine resulted from some impurities contained in the crude preparations used by Bacq. We

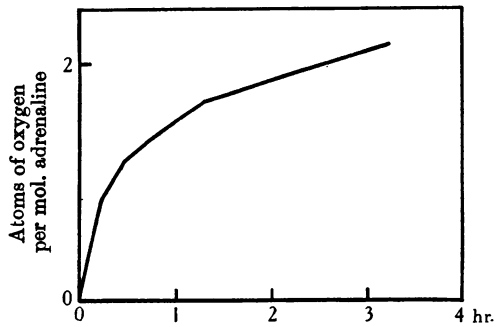


Fig. 2. Oxygen uptake of adrenaline with potato extract.

have therefore repeated the experiments with press juice of potatoes, but, apart from the fact that the oxygen uptake and the inactivation proceeded more slowly, not allowing a sharp differentiation between an initial rapid and delayed slow oxygen uptake, the results did not differ from those previously described. There was no formation of a depressor substance during the oxidation. The assay on the arterial blood pressure had to be made after atropine (1 mg. sulphate intravenously), since the press juice itself caused a fall of pressure on the non-atropinized cat, probably resulting from the presence of a choline ester in the extracts [Oury & Bacq, 1938]. Fig. 2 illustrates the oxygen uptake in an experiment with press juice of potatoes. The flasks were set up as follows:

Main flask: 1·6 c.c. phosphate buffer + 0·2 c.c. potato extract.
Side bulb: 0·2 c.c. adrenaline hydrochloride (=2 mg. base).
Inner tube: 0·3 c.c. *N* KOH.
$t = 20{\cdot}0°$; gas phase: air.

The curve of oxygen consumption shows a characteristic difference from that in Fig. 1. There is no sharp break in the curve after the uptake of 2 atoms; the rate of oxidation was in fact falling off prior to that point. In agreement with this finding were the results obtained for inactivation. There was no sharp point at which the activity disappeared. In the experiment of Fig. 2, for instance, 5 % of the initial activity was left after an incubation of 18 hr. The only conclusion that can be drawn from this experiment is that the clear-cut action of the purified enzyme is not to be seen, due to interference by other substances in the crude extracts.

Cytochrome c—*cytochrome oxidase.* The oxidation of adrenaline by polyphenol oxidase entails a reduction of the copper in the enzyme which in its turn is oxidized by molecular oxygen. In the oxidation of adrenaline by the cytochrome system there is involved a subsequent oxido-reduction of the three different cytochromes, and probably also of the oxidase before molecular oxygen is brought into reaction. The complexity of this mechanism may be responsible for the initial period of slow oxygen uptake. This period, during which the oxygen uptake slowly increased, was followed by a period of uniform oxidation rate which, however, remained below that observed with polyphenol oxidase. The oxygen uptake slowed down when 2 atoms of oxygen per mol. had been consumed. The break on the curve of Fig. 3 is less sharp than that of Fig. 1. In the experiment from which Fig. 3 is taken the manometer flasks were set up as follows:

Main flask: 1·0 c.c. phosphate buffer + 0·5 c.c. cytochrome *c* solution + 0·3 c.c. cytochrome oxidase.

Side bulb: 0·2 c.c. adrenaline hydrochloride (= 2 mg. base).

Inner tube: 0·3 c.c. *N* KOH.

$t = 19·0°$; gas phase : air.

Samples were assayed on the arterial blood pressure of the cat, the results being given in Table II. There was still 4 % adrenaline activity left after about 1 hr. of incubation when approximately 2 atoms of oxygen had been used. This can be understood if we consider that the reaction occurs more slowly than with polyphenol oxidase. A perceptible amount of the first reaction product must already have undergone further oxidation and contributed to the oxygen consumption when some of the adrenaline was still present as such.

Once the pressor activity had disappeared the solution remained inactive even if incubated for a further period of many hours. In no

instance did we observe a depressor activity. Similar results were obtained when the enzyme was incubated with an adrenaline solution 1 in 10^5.

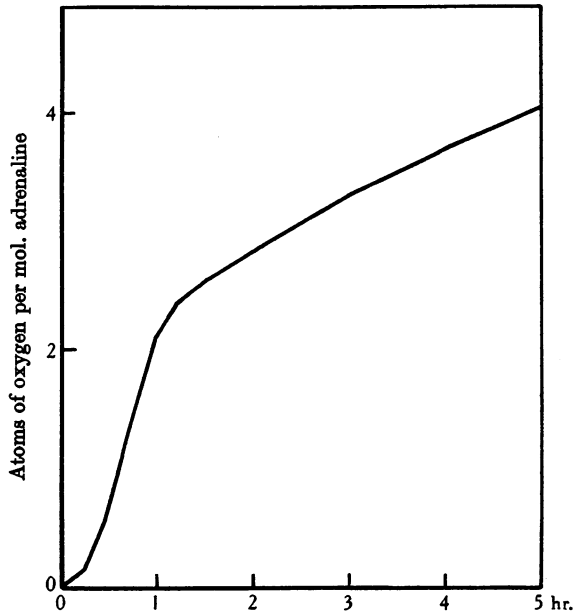


Fig. 3. Oxygen uptake of adrenaline with cytochrome + cytochrome oxidase.

TABLE II. Oxygen uptake and inactivation of adrenaline by cytochrome oxidase + cytochrome

Time of incubation in min.	Oxygen consumed in c.mm. O_2	Activity expressed in % of initial activity
51	196	25
63	271	4
175	367	0

Peroxidase. Since the oxidation does not involve an uptake of oxygen it cannot be measured manometrically. We have taken the colour changes as an approximate measure for the course of the reaction. As soon as the latter was started by tipping in the adrenaline from the side bulb the solution became pink. The intensity of the colour gradually increased and after 2 hr. the solutions were deep red. Later they became dark brown and a precipitate was formed. With the development of the red colour the solution gradually became inactive and remained so at later

stages when the change to the brown colour occurred. These results are illustrated in Table III, taken from an experiment in which the flasks were set up as follows:

Main flask: 1·6 c.c. phosphate buffer + 0·1 c.c. 0·4 *M* hydrogen peroxide (perhydrol) + 0·1 c.c. peroxidase solution (containing 0·3 mg. peroxidase).

Side bulb: 0·2 c.c. adrenaline hydrochloride (= 2 mg. base).

$t = 17·0°$; gas phase: N_2.

The assay in this experiment was done on the cat's blood pressure and on the isolated intestine of the rabbit. It will be seen that the loss of inhibitor activity on the intestine occurred at about the same time as

TABLE III. Inactivation of adrenaline by peroxidase

Time of incubation in min.	Colour	Activity expressed in % of initial activity	
		Cat's blood pressure	Rabbit's intestine
55	Pink	45	—
95	Red	—	7
135	Red	—	4
165	Red	—	0
191	Brownish	0	—

the loss of pressor activity. When the solution was retested after 3, 4 and 5 hr. on the cat it caused no change in blood pressure. There was no depressor activity. Similar results were obtained in experiments in which the initial adrenaline concentration was 1 in 10^5.

DISCUSSION

The curves of oxygen uptake in our experiments both with polyphenol oxidase and with the cytochrome system show that two different reactions can be distinguished. There is an initial fast reaction characterized by the development of a red colour, the uptake of 2 atoms of oxygen per mol. adrenaline, and the inactivation of the solution. This is followed by a prolonged reaction during which oxygen uptake is slow and the colour changes to brown. The initial fast reaction is the formation of adrenochrome which requires 2 atoms of oxygen [Green & Richter, 1937]. There is a parallelism between the amount of adrenaline inactivated and the formation of adrenochrome as measured by the oxygen uptake. The second slow reaction is probably not catalysed by the enzymes, adrenochrome being unstable in the presence of oxygen. This reaction does not

lead to new pharmacologically active oxidation products of adrenaline. The formation of adrenochrome by phenolases and its subsequent oxidation is therefore not responsible for the formation of Bacq's adrenoxine.

In the experiments with peroxidase no oxygen uptake could be measured, but the inactivation was also correlated with the formation of a red substance which is very likely adrenochrome. The experiments showed that the excitatory and inhibitory actions of adrenaline disappeared simultaneously. Similar results have been obtained in previous experiments in which adrenaline was incubated with extracts from mammalian liver [Blaschko & Schlossmann, 1936*b*]. We know now that in these experiments the inactivation of adrenaline must have been brought about mainly by amine oxidase. Thus, oxidation of the catechol group as well as that of the side chain is associated with simultaneous loss of the excitatory and inhibitory properties of the adrenaline molecule.

About the enzymic destruction of adrenaline *in vivo* little is known. Both amine oxidase and the phenolases may be involved. Some of the enzymic systems used in our experiments have not been found in higher animals. Peroxidase has been isolated from plants and polyphenol oxidase from plants and from invertebrates only. Recently, Mann & Keilin [1939] described the occurrence of copper-protein compounds in mammalian tissues. They were not catalytically active, but it is conceivable that similar compounds with phenolase activity exist. The cytochrome system is known to be present in mammalian cells and may participate in the destruction of adrenaline. In fact, the ubiquity of this system in the mammalian organism raises another question, i.e. the protection of adrenaline from the cytochrome system. This protection may be due to substances like ascorbic acid or sulphydryl compounds known to protect adrenaline from inactivation by phenolases *in vitro* [Toscano Rico & Malafaya Baptista, 1935; see also Richter, 1934]. The study of specific inhibitors of enzymes like choline esterase and amine oxidase has opened a new approach to the understanding of pharmacological problems. The inactivation of adrenaline is one of these problems. A recent observation of Clark & Raventós [1939] may find its explanation along these lines. They obtained prolonged action of adrenaline on the frog's auricle after ascorbic acid. Since the heart muscle contains the cytochrome system in high concentration the protection of adrenaline from inactivation by this system might explain the observation of Clark & Raventós.

SUMMARY

The inactivation of adrenaline by polyphenol oxidase, by cytochrome + cytochrome oxidase, and by peroxidase is studied in phosphate buffer *p*H 7·3.

(1) The oxygen uptake in the oxidation of adrenaline by polyphenol oxidase and by the cytochrome system is measured manometrically. There is an initial fast reaction in which 2 atoms of oxygen per mol. adrenaline are consumed and in which a red colour appears. There follows a second reaction, which is independent of an enzyme, during which the oxygen uptake is slow and the colour changes to brown. The initial reaction is the formation of adrenochrome from adrenaline and represents the inactivation of adrenaline. No new pharmacologically active substances are formed during the second reaction.

(2) The inactivation of adrenaline by peroxidase cannot be measured manometrically. Here, too, the inactivation occurs with the formation of a red substance, probably adrenochrome. In the inactivation adrenaline loses excitatory and inhibitory properties simultaneously.

(3) The formation of adrenochrome by phenolases and its subsequent oxidation is not associated with the formation of adrenoxine.

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