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THE INACTIVATION OF ADRENALINE

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In this paper experimental evidence is given of the existence of a system in mammalian tissues which actively destroys the biological activity of adrenaline.

Preliminary experiments showed that rat's liver tissue accelerated the inactivation of adrenaline in the presence of oxygen. The system responsible for this inactivation has been obtained from liver, kidney and intestine in the form of cell-free extracts. The purification and properties of these extracts and a number of observations on the mechanism of the inactivating reaction are described. The evidence brought forward leads to the conclusion that the inactivating system is a specific enzyme which catalyses the oxidation of adrenaline and that the inactivating reaction is different from other oxidation reactions of adrenaline hitherto described.

The fate of adrenaline in the animal body is unknown. The drug autoxidizes readily *in vitro* and it has therefore often been assumed that the same mechanism is responsible for the inactivation in the body. But it is unlikely that autoxidation is the only cause of the rapid inactivation *in vivo* as it has been observed that adrenaline is protected from autoxidation in the presence of certain tissues, tissue extracts, blood and other body fluids. The first observation of the protection of adrenaline was described by Oliver & Schäfer [1895] who found that small amounts of suprarenal extracts retained their activity very much longer in blood than in aqueous solution. This observation has frequently been confirmed. It is now known that in blood and tissues an inhibitor of autoxidation is present, and the role of different substances such as amino acids [Abderhalden & Gellhorn, 1923; Wiltshire, 1931], glutathione [Welch, 1934], ascorbic acid [Heard & Welch, 1935] and

guanidine [Burns & Secker, 1936] as inhibitors of autoxidation has been discussed.

The rate of disappearance of adrenaline is different in different organs; Elliott [1905] has shown that it does not disappear from the blood in its passage through the lungs, whereas it is removed from the circulation in the intestines and liver, as previously described by Carnot & Josserand [1902]. According to Trendelenburg [1929] and Pak [1926], the liver is of chief importance in the removal of adrenaline from the blood. They consider this removal to be due to the decomposition of the drug.

Elliott's statement, that "adrenaline disappears in the tissues which it excites", received some qualification through the findings of Pak [1926] in that he did not observe any appreciable removal of adrenaline from the blood in perfused hindlimbs. The question to what extent the various organs are active in removing adrenaline from the circulation calls for a more detailed examination.

Attempts to demonstrate the inactivation of adrenaline by isolated tissues *in vitro* have led to contradictory results. Embden & v. Fürth [1904] reported negative results when incubating tissue and tissue extracts with adrenaline, and Wiltshire [1931] observed even a protection of adrenaline in the presence of tissues. On the other hand, other observers claimed to have found inactivation in mammalian tissues and extracts. Langlois [1897] found inactivation in extracts of rabbit's liver and intestine. More recently, Toscano Rico & Baptista [1935*a*] found inactivation in extracts of the organs of various animals (see also Schütz, 1933; Koehler, 1934).

Methods

In studying the inactivation of adrenaline, tissue slices or extracts were incubated with adrenaline at 37° in Warburg manometer flasks of conical shape with one side bulb and an inner cup. This arrangement enabled the oxygen consumption to be measured. After suitable times the flasks were removed from the manometers and the remaining adrenaline activity was determined biologically.

The biological evaluation was made either on the isolated intestine of the rabbit or by the cat's blood-pressure method. The latter method is preferable for the evaluation of small amounts of adrenaline in the presence of tissue extracts, as in higher concentrations the extracts raise the tone of the intestine and make the response to adrenaline somewhat irregular.

The assay on rabbit's intestine was carried out with the experimental arrangement described elsewhere by one of us [Schlossmann, 1934]. The evaluation by cat's blood pressure was done after an injection of 2 c.c. of a 5 p.c. novocaine solution through the membrana atlanto-occipitalis. The assay was always carried out immediately after the removal of the flasks from the thermostat; the possible loss in activity before making the estimation was generally negligible. It was found that further inactivation could be prevented by acidifying with hydrochloric acid; but in the experiments described this precaution was unnecessary.

The adrenaline activities are recorded as percentages of the adrenaline concentration initially present.

Results

Experiments with tissue slices

In a series of preliminary experiments the rates of adrenaline disappearance in the presence and in the absence of slices of rat's liver tissue were compared. In these experiments an acceleration of adrenaline inactivation in the presence of tissue was observed. This acceleration occurred only in the presence of oxygen; in nitrogen no inactivation was found either in the presence or absence of tissue.

The tissue slice method, however, was not found to be suitable for the quantitative study of adrenaline inactivation. The rate of adrenaline disappearance in buffer solution cannot justifiably be compared with that in the presence of tissue, as with the tissue an unknown amount of autoxidation inhibitor is added. The inhibition of the autoxidation may even counterbalance any destruction of adrenaline by the tissue. Although the latter was not the case in our experiments, it is possible that it was so in Embden & v. Fürth's [1904] and Wiltshire's [1931] negative experiments. Bain & Suffolk [1936] have tried to avoid this difficulty by following the inactivation in blood with and without liver tissue.

A second reason for abandoning the tissue slice method was that the respiration of the tissue was too large to allow an accurate measurement of any increased oxygen uptake due to adrenaline.

Oxidation of adrenaline in liver extracts

The attempt was therefore made to obtain from rat's liver an extract which accelerated the oxidative inactivation of adrenaline. Active extracts were prepared which were free from cells but contained granular

cell fragments. These extracts had a certain spontaneous oxygen uptake. On addition of adrenaline the oxygen uptake was greatly increased, as is shown by the following experiment:

Oxidation of adrenaline in liver extracts. Adrenaline 1 : 1000. 37°; in O_2.

10 g. rat's liver ground thoroughly with sand in a mortar, 20 c.c. *M*/15 phosphate buffer *p*H 7·3 added, centrifuged for 5 min.; the turbid supernatant fluid used as extract.

In all later experiments the extracts were prepared as here described.

Two manometer flasks were set up as follows:

Flask No. ...	1	2
Main vessel	1·8 c.c. extract	1·8 c.c. extract
Side bulb	0·2 c.c. water	0·2 c.c. adrenaline solution 1 : 100
Inner cup	0·3 c.c. *N* KOH	0·3 c.c. *N* KOH

The flasks were incubated at 37°, and after 85 min. the contents of the side bulbs were tipped into the main flasks.

The oxygen uptakes are shown in Fig. 1. The difference between the readings in flasks 2 and 1 is shown in Fig. 2; this difference represents the additional oxygen uptake due to adrenaline. The subsequent figures show only the additional oxygen uptakes obtained in this way.

Fig. 2 shows the characteristic course of the oxygen consumption due to adrenaline. It can be seen that the rate of oxygen uptake increased some time after the beginning of the experiment. In the experiment shown the total additional oxygen uptake was about $3\frac{1}{2}$ atoms of oxygen per molecule of adrenaline, and in a number of similar experiments it was between $3\frac{1}{2}$ and $4\frac{1}{2}$ atoms.

Biological determinations of the adrenaline activity showed that all activity had disappeared when the additional oxygen uptake had come to an end. In order to relate the inactivation of the drug to the oxygen uptake, the reaction was allowed to take place for different lengths of time in a number of experiments, and the remaining activity was determined biologically after the oxygen uptake had been measured.

The result of these experiments is shown in Fig. 3, in which the remaining adrenaline activity is plotted against the oxygen uptake. It can be seen that inactivation was always complete when 2 atoms of oxygen per molecule of adrenaline had been taken up. Since the total oxygen uptake was about 4 atoms of oxygen per molecule, it follows that the oxygen used in the later part of the reaction oxidized not adrenaline itself, but the biologically inactive oxidation products of adrenaline.

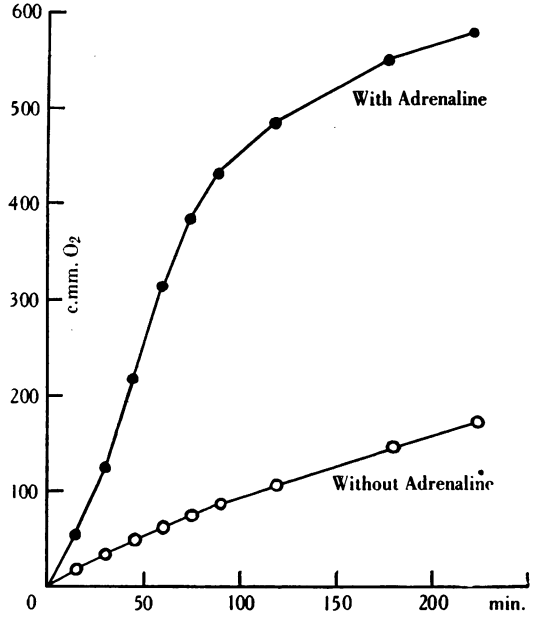

Fig. 1. Oxygen uptake of rat's liver extract with and without adrenaline 1 : 1000.

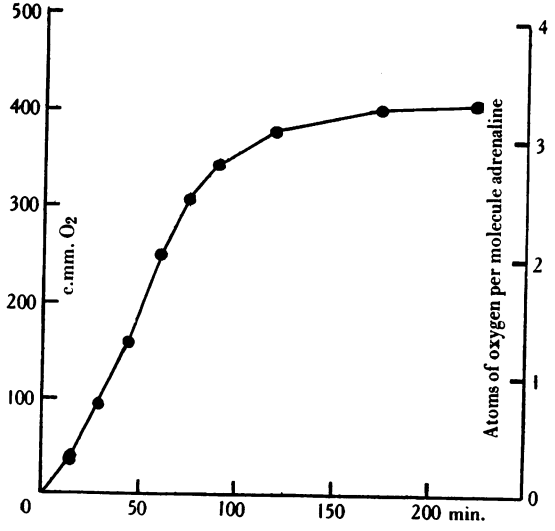

Fig. 2. Same experiment as in Fig. 1. Additional oxygen uptake due to adrenaline.

Oxidation in liver extracts and autoxidation

The autoxidation of adrenaline in buffer solutions shows characteristic differences from the oxidation in liver extracts just described. In rat's liver extract the total additional oxygen uptake was about 4 atoms of oxygen per molecule of adrenaline. In experiments in which adrenaline was incubated in phosphate buffer solution (*p*H 7·3) only, the total oxygen uptake was found to be about 7 atoms of oxygen per molecule

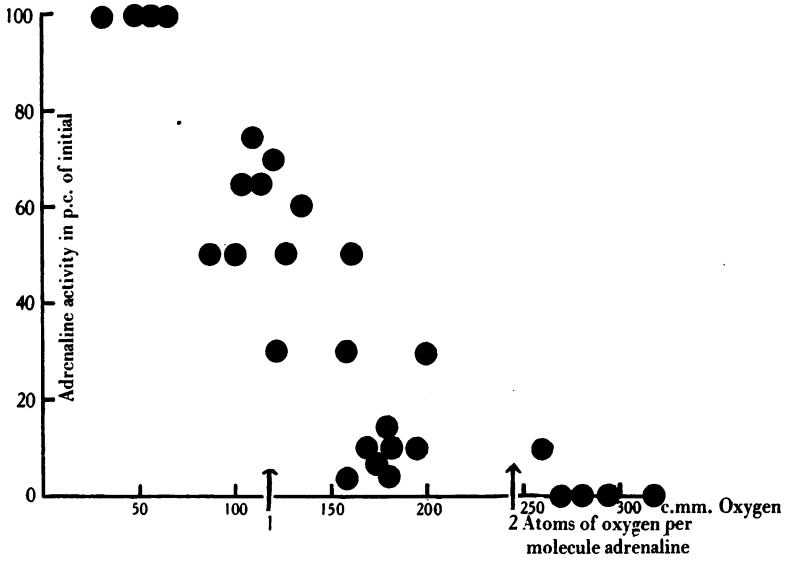

Fig. 3. Relation between additional oxygen uptake and inactivation of adrenaline in rat liver extracts. Adrenaline 1 : 1000.

of adrenaline. Dulière & Raper [1930] found 6·14 atoms of oxygen in a similar experiment at *p*H 8·0, and Welch [1934] reported an oxygen uptake of 7–8 atoms per molecule of adrenaline.

A difference was also found in the relationship between inactivation and oxygen uptake. Adrenaline was incubated in *M*/15 phosphate buffer solution (*p*H 7·3) and the oxygen uptake was measured manometrically. The incubation was then interrupted at different stages of the reaction and the remaining adrenaline activity was determined biologically. A comparison of Fig. 4 with Fig. 3 shows that a larger

amount of oxygen was required to inactivate a given amount of adrenaline in the autoxidation of adrenaline than in the oxidation in the presence of extracts.

The characteristic differences of adrenaline oxidation in the presence and absence of liver extracts left no doubt that the mechanism of the two reactions was different. But it appeared probable that some

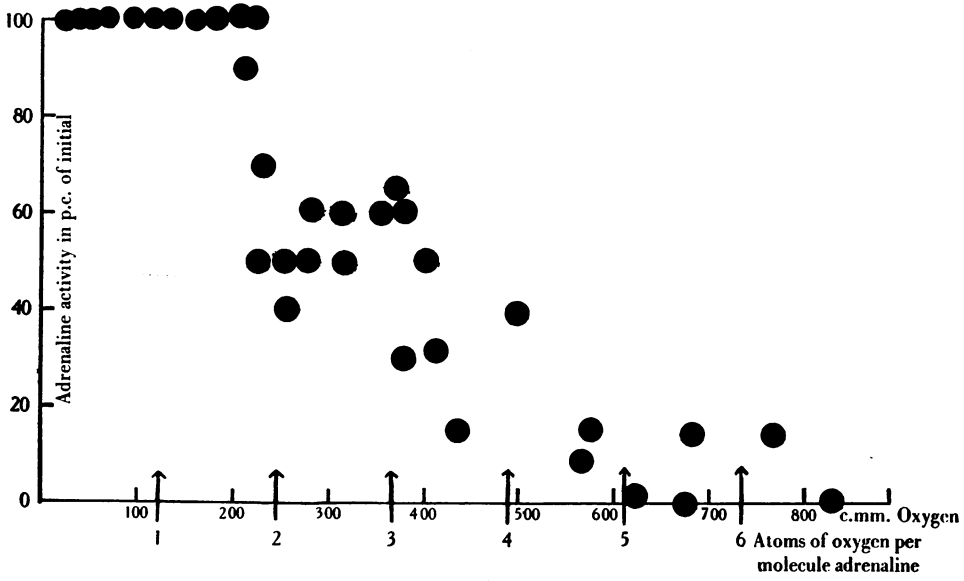

Fig. 4. Relation between oxygen uptake and inactivation of adrenaline in phosphate buffer solution *p*H 7·3. Adrenaline 1 : 1000.

autoxidation also occurred in the presence of extracts. This was made likely by the shape of the curve shown in Fig. 2 and by the observation that in the extracts, as in the autoxidation of adrenaline, coloured oxidation products were formed.

Effect of cyanide

Attempts were therefore made to exclude autoxidation and secondary reactions by adding cyanide to the extracts. That autoxidation was effectively prevented by this means was shown by the fact that no colour developed.

In the presence of cyanide, the oxidative inactivation of adrenaline

involved the uptake of only 1 atom of oxygen. A typical experiment follows:

Oxidation of adrenaline in rat's liver extract with and without 10^{-3} *M* HCN. 37°; in O_2. Four manometer flasks were set up as follows:

Flask No. ...	1	2	3	4
Main vessel	1·7 c.c. extract +0·1 c.c. water	1·7 c.c. extract +0·1 c.c. water	1·7 c.c. extract +0·1 c.c. *M*/50 HCN solution	1·7 c.c. extract +0·1 c.c. *M*/50 HCN solution
Side bulb	0·2 c.c. water	0·2 c.c. adrenaline solution 1:100	0·2 c.c. water	0·2 c.c. adrenaline solution 1:100
Inner cup	0·3 c.c. *N* KOH	0·3 c.c. *N* KOH	0·3 c.c. KOH–KCN*	0·3 c.c. KOH–KCN*

* KOH–KCN mixture according to Krebs [1935].

Initial adrenaline concentration in flasks 2 and 4, 1 : 1000; cyanide concentration in flasks 3 and 4, *M*/1000.

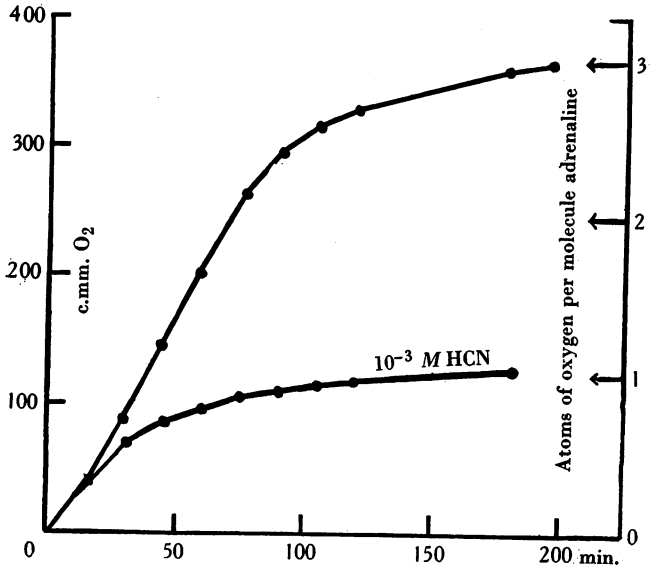

Fig. 5. Additional oxygen uptake due to adrenaline with and without 10^{-3} *M* HCN. Adrenaline 1 : 1000.

Fig. 5 shows the differences between the oxygen uptakes in flasks 2 and 1 and in flasks 4 and 3 respectively.

It can be seen that the rates of adrenaline oxidation were at first identical with and without cyanide, but that after a short time a difference developed. While the rate of oxygen uptake without cyanide remained constant or slightly increased, in the presence of cyanide the additional oxygen uptake slowed down and came to a standstill when 1 atom of

oxygen per molecule of adrenaline had been taken up. That all the adrenaline had then reacted was shown by biological determination. The inactivation during the course of the reaction was proportional to the oxygen uptake. This is shown in Fig. 6.

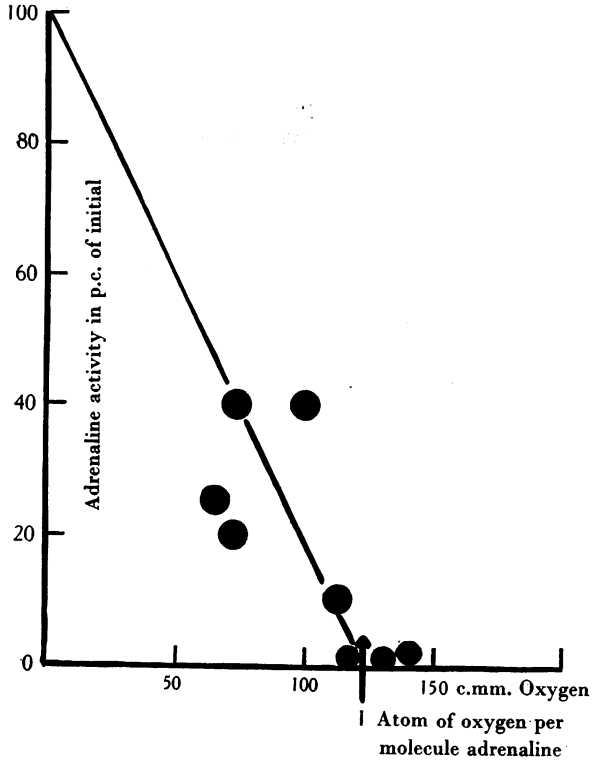

Fig. 6. Relation between additional oxygen uptake and inactivation of adrenaline in 10^{-3} *M* HCN. Adrenaline 1 : 1000.

It was confirmed in many experiments that in cyanide the increased oxygen uptake of the extracts stopped when 1 atom of oxygen per molecule of adrenaline had been taken up. This was not due to the inactivation or inhibition of the active system as was shown by adding more adrenaline, when the increased oxygen uptake recommenced.

These experiments show that rat's liver contains a cyanide-insensitive system that inactivates adrenaline in a reaction in the course of which 1 atom of oxygen per molecule of adrenaline is consumed. The relation between inactivation and oxygen uptake should therefore be represented by the straight line drawn in Fig. 6; the points giving the experi-

mental results show a satisfactory agreement. No conclusions are drawn from these experiments as to the nature of the cyanide-sensitive oxidation.

Distribution of the inactivating system

The characteristic course of adrenaline oxidation in the presence of cyanide gave a criterion that made it possible to find out where the inactivating system described was present and to distinguish the reaction catalysed by it from other oxidative reactions of adrenaline occurring in tissue extracts. It was assumed that the system was present in those extracts in which the oxygen uptake in 10^{-3} *M* HCN was 1 atom of oxygen per molecule of adrenaline. By applying this test, the system was found in extracts from liver and kidney of all the animals so far gxamined—rat, guinea-pig and rabbit. The activity of liver tissue was ereater than that of kidney tissue; guinea-pig's organs contained relatively more activity than those of the rat and rabbit, but a survey of the distribution of the inactivating system remains to be carried out. Besides being present in liver and kidney, the system was found in extracts from guinea-pig's small intestine; it was not present—at least in concentrations comparable to those found in these three organs—in skeletal muscle or in spleen.

The data given demonstrate that the system was capable of inactivating a considerable amount of adrenaline in relatively short time: it can be calculated that in the experiment recorded in Fig. 5 the extract from 1 g. of rat's liver was capable of inactivating 1 mg. adrenaline in 15 min. The activity of guinea-pig's liver was still greater: 1 g. of tissue inactivated 1 mg. adrenaline in 8 min.

Purification and properties of the system

The experimental evidence so far reported made it likely that the system responsible for the inactivation was an enzyme. Experiments were therefore undertaken to purify the system and to study its properties. The crude extract contained many substances that were possible substrates for oxidation, partly dialysable (amino acids, lower carbohydrates) and partly non-dialysable (glycogen). It was therefore desirable to remove these substances in order to reduce the spontaneous oxygen consumption of the extracts. In the following experiment liver extract was purified by dialysis and the glycogen was removed by digesting with takadiastase.

20 c.c. guinea-pig's liver extract were digested with 0·04 g. taka-diastase for 25 min. at 35°, and then dialysed overnight. The specimen prepared in this way was found by Molisch's test, after removing protein, to be carbohydrate-free. This preparation oxidized adrenaline in the same way as the crude extracts.

It was also found possible to purify the system by precipitation with 30 p.c. alcohol. The system prepared in this way consisted of a nearly colourless protein suspension. It kept its activity for several days at 0°, but was completely inactivated by heating for 5 min. at 80°. A specimen prepared in this manner containing 0·073 g. dry weight in 2 c.c. gave a spontaneous oxygen uptake of only 20 c.mm. in 2 hours at 37°, and oxidized 1 mg. of adrenaline in 25 min. under the conditions described above.

Inhibition of adrenaline autoxidation

When it was attempted to purify the preparations further by adsorption on kaolin it was found that with preparations purified beyond a certain point autoxidation of adrenaline set in even in the presence of cyanide: the oxygen uptake became abnormally high and coloured oxidation products were formed.

It was clear that in the original extracts some other factor together with cyanide was inhibiting the autoxidation of adrenaline, and it appeared probable that the reducing substances such as ascorbic acid and sulphydryl compounds in the extracts were partly responsible.

The question of the identity of the autoxidation inhibitor in tissue extracts lies outside the scope of the present investigation; but experiments on the autoxidation of adrenaline in buffer solution showed that whereas the autoxidation was inhibited comparatively little by 10^{-3} *M* cyanide alone, it was inhibited very strongly by the combined action of HCN + glutathione. HCN + ascorbic acid was less effective, but HCN + cysteine inhibited strongly (see Fig. 7). These observations are in agreement with the findings of Heard & Welch [1935], who also observed a protection of adrenaline by glutathione, ascorbic acid, and cysteine.

These experiments suggest that in the oxidation of adrenaline catalysed by tissue extracts in the presence of cyanide, the cyanide acts by protecting the autoxidation inhibitors of the tissue rather than by inhibiting the autoxidation of adrenaline directly.

The effect of inhibitors

The effect of cyanide has already been described. Carbon monoxide was also tested. In a gas mixture containing 80 p.c. CO + 20 p.c. O_2 the rate of oxidation of adrenaline in the presence of guinea-pig's liver extracts was the same as that in air.

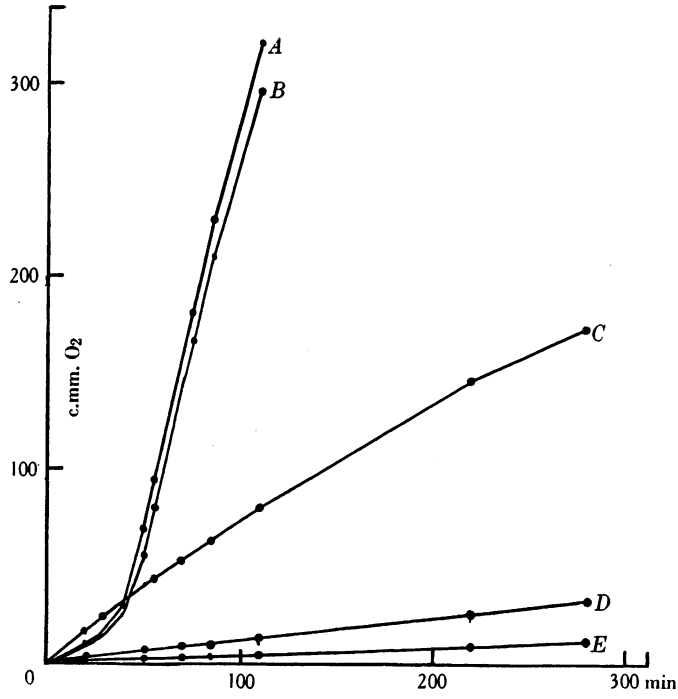

Fig. 7. Inhibition of autoxidation of adrenaline in phosphate buffer solution *p*H 7·3.

A. 2 c.c. phosphate buffer + adrenaline 1 : 1000.

B. 2 c.c. phosphate buffer + adrenaline 1 : 1000 + *M*/1000 HCN.

C. 2 c.c. phosphate buffer + adrenaline 1 : 1000 + *M*/1000 HCN + 2 mg. ascorbic acid.

D. 2 c.c. phosphate buffer + adrenaline 1 : 1000 + *M*/1000 HCN + 1·6 mg. cysteine hydrochloride.

E. 2 c.c. phosphate buffer + adrenaline 1 : 1000 + *M*/1000 HCN + 4 mg. reduced glutathione.

In the presence of octyl alcohol the oxidation of adrenaline was strongly inhibited. Ethyl urethane also inhibited the cyanide-insensitive oxidation of adrenaline in extracts from guinea-pig's and rat's liver; glutathione did not inhibit.

Influence of pH

The autoxidation inhibitor in the extracts was not effective above pH 8·0. Autoxidation then became significant even in the presence of cyanide, and prevented observation beyond this value. Up to pH 8·0 the activity of the preparation from rat's liver was found to vary with the pH in the manner shown in Fig. 8.

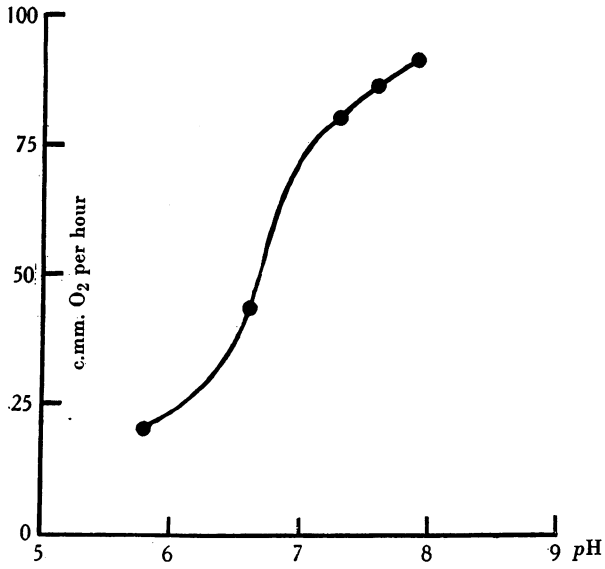

Fig. 8. Effect of pH on the additional oxygen uptake in liver extract.

Oxidation of l-(−)-*adrenaline and* d-(+)-*adrenaline*

A comparison of the oxidation rates of the two stereoisomers of adrenaline showed a marked difference. A typical experiment is shown in Fig. 9. The naturally occurring lævorotatory compound was oxidized at a rate twice that of the dextrorotatory substance. In the absence of cyanide the initial difference in the reaction rates disappeared after some time probably because secondary reactions such as autoxidation, which were not stereospecific, set in. Extract from rat's kidney showed the same stereospecificity as those from liver.

Other substrates

Epinine, $C_6H_3(OH)_2CH_2CH_2NHCH_3$, was oxidized by the extracts in a similar manner to adrenaline. Fig. 10 gives an experiment with rat's liver extract. It can be seen that the total additional oxygen uptake in

the absence of cyanide was less than for adrenaline, only about 2 atoms of oxygen per molecule of epinine—but in the presence of 10^{-3} *M* HCN about 1 atom of oxygen per molecule was taken up.

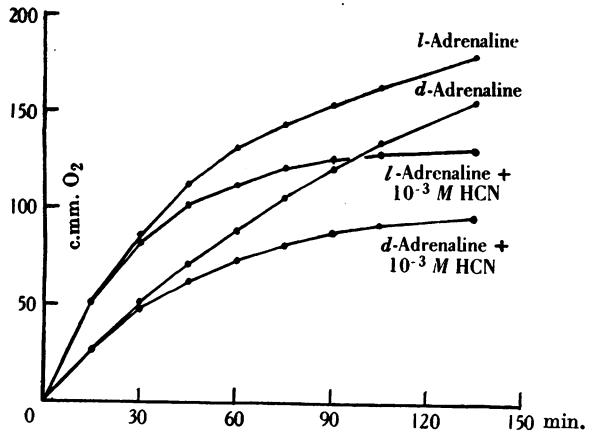

Fig. 9. Oxidation of *l*-(−)-adrenaline and *d*-(+)-adrenaline in dialysed extract from guinea-pig's liver with and without 10^{-3} *M* HCN. (After 270 min. the oxygen uptake of *d*-adrenaline in the presence of cyanide was approximately 1 atom of oxygen per molecule of adrenaline.)

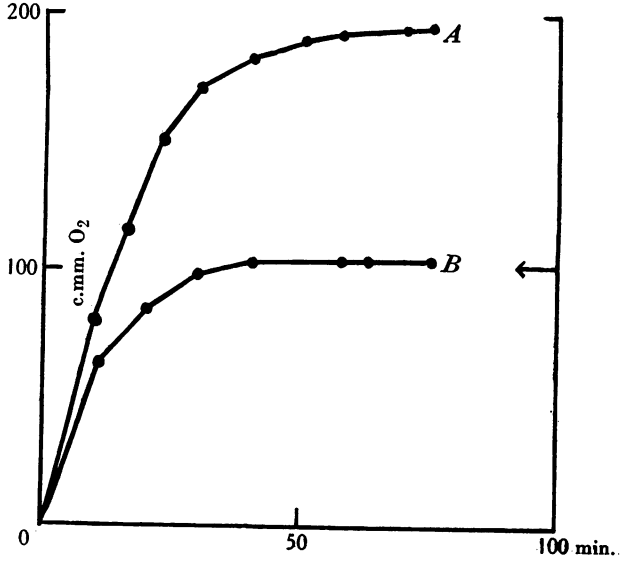

Fig. 10. Epinine oxidation in liver extract with and without 10^{-3} *M* HCN. (Arrow on right margin denotes one atom of oxygen per molecule of epinine.)

Adrenalone[1], $C_6H_3(OH)_2COCH_2NHCH_3$, behaved in a very different manner from adrenaline. The rate of oxidation was greater with cyanide than without and the reaction with cyanide proceeded beyond 1 atom of oxygen per molecule. In this case the possibility of a chemical reaction between adrenalone and cyanide must be considered.

Experiments with other adrenaline derivatives will be reported in another paper.

Reaction product

Observations on the chemistry of the reaction product will be reported later, but it may be mentioned that the biologically inactive reaction product still gave the green coloration with ferric chloride characteristic of a catechol derivative.

Discussion

The adrenaline inactivating system in liver extracts had the normal characters of an enzyme: it was thermolabile, it was non-dialysable, it was sensitive to narcotics and it showed a marked stereospecificity.

The action of inhibitors on the adrenaline oxidase distinguishes this enzyme from catechol oxidase, another enzyme known to oxidize adrenaline. The main differences are:

(*a*) Catechol oxidase is poisoned by 10^{-3} HCN and CO [Keilin, 1928], whereas adrenaline is not affected by these inhibitors.

(*b*) Catechol oxidase is not inhibited by narcotics [Richter, 1934], whereas adrenaline oxidase is inhibited.

(*c*) Catechol oxidase is known to be inhibited by sulphydryl compounds [Richter, 1934], and Toscano Rico & Baptista [1935*b*] have demonstrated that the enzymic oxidation of adrenaline by potato juice is inhibited by reduced glutathione: the adrenaline oxidase is not affected by these compounds.

That the product of the reaction was colourless and still gave a green colour with ferric chloride showed that the reaction is not identical with that catalysed by catechol oxidase.

The question arises whether the system studied in these experiments is responsible for inactivation of adrenaline in the living animal. There is no known reason why the system should not be active *in vivo*. It is suggestive that the organs in which the system has so far been found include those which, according to Trendelenburg [1929], are mainly responsible for the removal of the drug from the circulation.

[1] We are gratefully indebted to the I.-G. Farbenindustrie, Frankfurt a./Main, for the gift of adrenalone.

While it seems justifiable to ascribe some function to the system, it may not be the only mechanism concerned in the oxidation of adrenaline *in vivo*. The experiments related above demonstrate that in tissue extracts at least two different kinds of oxidation can be distinguished, namely the enzymic oxidation described, and secondly an oxidation which resembles the autoxidation in buffer solution. The separation of these two different types of reaction was effected by making use of their different sensitivity to cyanide.

The present paper does not deal with the cyanide-sensitive oxidation. It remains to be investigated whether this reaction is only autoxidation or whether an enzymic factor is also concerned. The extracts of spleen and muscle in which the adrenaline oxidase was not found in significant amounts also oxidized adrenaline, but this reaction was inhibited by cyanide. It is possible that part of this oxidation was due to a catechol oxidase the occurrence of which in mammalian tissues has recently been made likely by Keilin & Hartree [1936]. Substances found to act as inhibitors of the non-specific adrenaline oxidation in the extracts are at the same time inhibitors of catechol oxidase, and the possibility cannot be excluded that both autoxidation and oxidation by a catechol oxidase were simultaneously inhibited in our experiments.

Whether the non-specific oxidation of adrenaline is responsible for any inactivation of the drug *in vivo* is doubtful in view of the fact that in the body the presence of reduced sulphydryl compounds should inhibit this type of reaction. This question had already been discussed by Welch [1934] and by Toscano Rico & Baptista [1935*b*]. On the other hand it has been shown that the system described in this paper is active under these conditions.

Summary

1. Slices of rat's liver tissue accelerated the inactivation of adrenaline in the presence of oxygen.

2. Extracts from liver, kidney and intestine of rats, guinea-pigs and rabbits showed an increased oxygen uptake on the addition of adrenaline.

3. The oxidation of adrenaline in these extracts in the presence of cyanide involved the uptake of 1 atom of oxygen per molecule of adrenaline; the adrenaline inactivation under these conditions was proportional to the oxygen uptake.

4. The cyanide-insensitive oxidation of adrenaline in these extracts was inhibited by narcotics. It was not inhibited by carbon monoxide or glutathione.

5. The purification of the inactivating system is described; the system was non-dialysable and thermolabile.

6. The system oxidized *l*-(−)-adrenaline twice as fast as *d*-(+)-adrenaline.

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