The Formation of Hydrogen Carriers by Haematin-catalyzed Peroxidations

2. SOME REACTIONS OF ADRENALINE AND ADRENOCHROME

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(*Received* 18 *August* 1948)

Green & Richter (1937) found that adrenaline induces a vigorous oxygen uptake when added in low concentrations to the reconstructed lactic and malic dehydrogenase systems of heart muscle. This effect was found to be due to the oxidative formation from adrenaline of a substance which they identified and named adrenochrome. The formation of adrenochrome depended on the presence in their enzyme preparations of a factor which they considered very probably to be cytochrome *c*. It was further found that in the presence of cytochrome *c* the effect was stimulated by hydrogen peroxide. They assumed that the role of hydrogen peroxide was the oxidation of ferrocytochrome *c*, and that ferricytochrome *c* oxidized adrenaline to adrenochrome. Cytochrome *c* and hydrogen peroxide thus exerted what Green & Richter called a 'trigger' effect, evidently by hastening the formation of adrenochrome. The addition of low concentrations of adrenochrome itself to the enzyme-adrenaline system also caused a 'trigger' effect, the reaction lag being shortened, but the maximum rate eventually reached not being much affected.

It was established that, once formed, the adrenochrome acted as a carrier in these systems. Green & Richter were not certain whether the reoxidation of the reduced (leuco) adrenochrome was enzymic or autoxidative.

Hermann, Boss & Friedenwald (1946) have shown that 'oxidized adrenaline' solutions are able to take part in a coupled oxidation of ascorbic acid, and that the rate in this case is further stimulated by the addition of cytochrome *c*.

In an earlier paper (Albert & Falk, 1949) the peroxidative oxidation of certain acridine and quinoline compounds, catalyzed by cytochrome *c* or methaemoglobin, was discussed. In the present paper a similar peroxidative oxidation of adrenaline is described; adrenochrome is shown to act as an autoxidizable hydrogen carrier in the oxidation of ascorbic acid, and to catalyze the oxidation of adrenaline itself. These reactions offer a possible explanation of the 'trigger effects' of cytochrome *c* and adrenochrome described by Green & Richter (1937). They are a further example of the non-specific haematin peroxidative effect, which may also be of importance *in vivo*.

EXPERIMENTAL

Materials

Water for all purposes was double distilled from an all glass still. Adrenaline was pure (−)-adrenaline, m.p. 213° (decomp.), kindly presented by Burroughs, Wellcome and Co., Sydney. It was stored under N_2, and weighed amounts were dissolved immediately before use in 1 equiv. of 0·1 N-HCl and made up to the required volume.

Adrenochrome was prepared from adrenaline by the method of MacCarthy (1945, 1946), quoted by Randall (1947); melting point, after crystallizing from dry methanol-formic acid, was 130° (decomp.). (Found: C, 60·1; H, 5·1; N, 7·6. Calc. for $C_9H_9O_3N$: C, 60·3; H, 5·1; N, 7·8%.) The monoxime of this adrenochrome was prepared and, as reported by Veer (1942*a*), it was found that its melting point rose as the substance lost water. After drying quickly on porous tile it melted sharply, with decomposition, at 175° and at 180° (decomp.) after drying *in vacuo* over $CaCl_2$. Solutions of adrenochrome in water were prepared immediately before use.

The ascorbic acid was pure; solutions were made in distilled water, with the addition of 1 equiv. of 0·1 N-NaOH, immediately before use. H_2O_2 solutions were prepared by diluting 100 vol. peroxide. Schering-Kahlbaum copper-free cysteine was used. The melting point of the quinol was 286°, and that of the catechol, 104°. The cytochrome *c*, methaemoglobin and catalase were prepared as described previously (Albert & Falk, 1949).

Methods

O_2 uptakes were measured in Warburg respirometers at pH 7·0 and 25 or 37·5°. The gas phase was usually air, and the shaking rate 120 per min. The total volume of liquid per flask was 2·0 ml., and the final molarity of the phosphate buffer was 0·12.

RESULTS

The effect of haematin compounds on the oxidation of adrenaline

Fig. 1 shows the effect of cytochrome *c* on the rate of oxidation of adrenaline solutions at pH 7 and 37·5°. The rate gradually increased in all cases to

a maximum, which was maintained steadily for some time; the lag, however, was decreased in proportion to the amount of cytochrome *c* added. In these experiments the red colour, characteristic of adrenochrome, appeared at about the end of the lag.

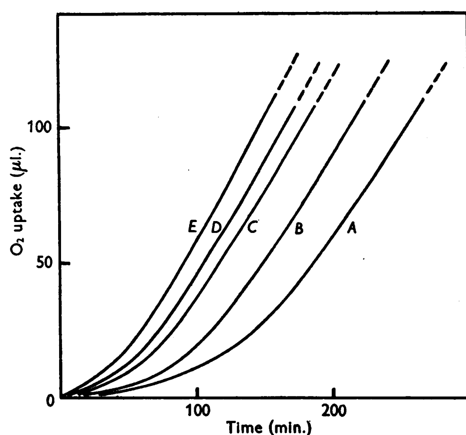


Fig. 1. The effect of cytochrome *c* on the oxidation of adrenaline. O_2 uptake was measured at pH 7 and 37·5°. Each flask contained 2·0 mg. of adrenaline. Cytochrome *c* in curves *A–E*; 0, 0·1, 0·2, 0·3 and 0·5 mg. respectively.

After 10 hr. oxygen uptake was still occurring in all the flasks at rates only slightly lower than the maximum. At this time, the depth of the colour was greater the more cytochrome *c* had been added. Finally the colour became very dark, suggesting melanin formation. There was no output of carbon dioxide. The adrenaline was eventually oxidized past adrenochrome, even in the absence of cytochrome *c* (theoretical oxygen uptake for 1 mg. adrenaline to adrenochrome, 122 µl.).

Table 1. *Peroxidative catalysis of the oxidation of adrenaline*

(O_2 uptakes were measured at pH 7 and 37·5°. Each flask contained 2·0 mg. adrenaline ($5·6 \times 10^{-3}$M). Sufficient catalase was added to decompose completely 2·0 ml. of M-H_2O_2 in 10 min.; the cytochrome *c* was $0·9 \times 10^{-5}$M, the methaemoglobin $0·22 \times 10^{-5}$M, and the H_2O_2 10^{-3}M. The lag (time to reach maximum rate) was reproducible to ±3 min.)

Reagents added	Lag (min.)
—	180
H_2O_2	180
Cytochrome *c*	65
Cytochrome *c* + H_2O_2	30
Cytochrome *c* + catalase	140
Methaemoglobin	75
Methaemoglobin + H_2O_2	35
Methaemoglobin + catalase	170

Methaemoglobin reacted similarly to cytochrome *c* (Table 1). In both cases the further stimulation (shortening of lag) by hydrogen peroxide and the inhibition by catalase suggested a peroxidative mechanism.

Green & Richter (1937) found a very rapid uptake of oxygen in the system adrenaline-cytochrome *c*-cytochrome oxidase. Here the adrenaline was simply the substrate for the cytochrome system; when the oxidase was boiled there was no uptake in the first 5 min. The peroxidative effect described above is only revealed when measurements are made for longer periods.

The effect of adrenochrome on the oxidation of adrenaline

Green & Richter (1937) studied the system malate, malic dehydrogenase, coenzyme, cyanide and adrenaline. In the full system small amounts of adrenochrome greatly stimulated the rate of oxygen uptake and the oxidation of adrenaline to adrenochrome. In the absence of malate they found that the addition of adrenochrome did not stimulate the oxygen uptake (during the first 3 min.) and did not bring about the oxidation of adrenaline to adrenochrome.

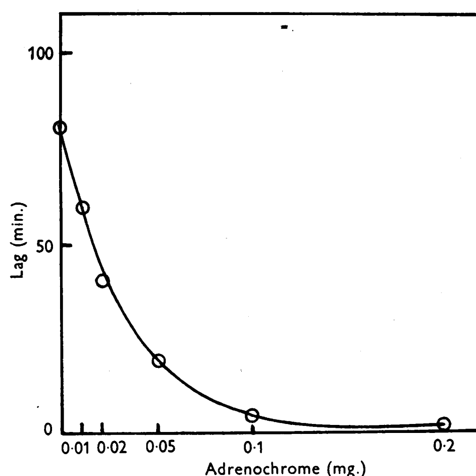


Fig. 2. The effect of adrenochrome on the oxidation of adrenaline. O_2 uptake measured at pH 7 and 37·5°. Each flask contained in the main bulb 2·0 mg. of adrenaline, and the adrenochrome solutions were tipped from a side arm. The adrenaline was $5·6 \times 10^{-3}$M (0·2 mg. adrenochrome gave a molarity of $5·4 \times 10^{-4}$).

It is now found that adrenochrome shortens the lag in the oxidation of adrenaline in a simple system containing only adrenaline and adrenochrome at pH 7 and 37·5°. The results are shown in Fig. 2; the lag was progressively shortened with increasing amounts of adrenochrome. In controls with adrenochrome alone, the oxygen uptake was negligible. The original oxygen-uptake curves were

of the same type as those shown in Fig. 1, and only with very high adrenochrome concentrations could any stimulation be observed in the first 3 min.

Adrenochrome did not increase the rate of autoxidation of cysteine, catechol or quinol at pH 7 and 25° or 37·5°.

The effect of adrenochrome on the oxidation of ascorbic acid

Adrenochrome catalyzes the oxidation of ascorbic acid (Fig. 3). In these experiments there was no lag, and the oxygen uptake was linear within certain limits of time.

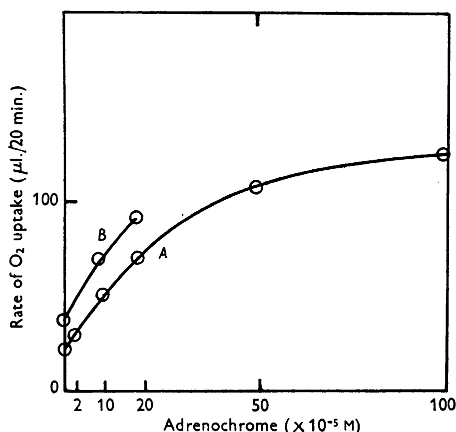


Fig. 3. The effect of adrenochrome on the oxidation of ascorbic acid. O_2 uptake was measured at pH 7. Each flask contained in the main bulb 1·0 mg. ascorbic acid ($2·8 \times 10^{-3}$ M). The adrenochrome was tipped from a side arm. Curve *A*, 25°; curve *B*, 37·5°.

Hermann *et al.* (1946) found that 'oxidized adrenaline' solutions had a similar effect on the oxidation of ascorbic acid, and that a further stimulation was caused by cytochrome *c*. The present author has repeated this experiment under the conditions described by Hermann *et al.* (1946) with quantitative agreement with their results. These workers considered that the 'oxidized adrenaline' solutions contained 'some unoxidized adrenaline and several oxidation products at different steps towards the maximum possible oxidation'. In this connexion it is noteworthy that with an amount of this solution (prepared according to their method) equivalent to an original 0·4 mg. of adrenaline, 3·0 mg. of ascorbic acid absorbed only 72 μl. of oxygen in 20 min. at pH 7·0 and 37·5°. Under similar conditions, but with 0·4 mg. of adrenochrome instead of oxidized adrenaline, 280 μl. of oxygen were absorbed.

Under no conditions, however, was cytochrome *c* found to stimulate the rate of oxidation of ascorbic acid in the presence of pure adrenochrome alone. As has been shown above, cytochrome *c* considerably stimulates the oxidation of adrenaline, and it seemed possible that the cytochrome *c* stimulation of Hermann *et al.* (1946) was due to the effect of the cytochrome *c* in converting unchanged adrenaline in the solution of 'oxidized adrenaline' to adrenochrome. Provided that the rate was limited by the adrenochrome concentration (cf. Fig. 3) cytochrome *c* should thus cause a stimulation. Under certain conditions (Table 2) it was in fact found that cytochrome *c* caused a stimulation in the rate of oxidation of ascorbic acid in the presence of mixtures of adrenochrome and adrenaline.

Table 2. *Oxidation of ascorbic acid with a mixture of adrenaline and adrenochrome*

(O_2 uptakes were measured at pH 7 and 37·5°. Each flask contained 2·0 mg. of ascorbic acid in a side arm, the remainder of the reagents being in the main bulb. Adrenochrome was limiting in Exps. 1 and 2. All values are corrected for ascorbate autoxidation (15 μl./10 min.). In Exps. 1 and 2, the flasks were shaken in the bath for 30 min. before tipping in the ascorbic acid.)

	Exp. 1	Exp. 2	Exp. 3	Exp. 4
Adrenaline (mg.)	0·3	0·3	0	0
Adrenochrome (mg.)	0·036	0·036	0	0
Cytochrome *c* (mg.)	0	0·5	0	0·5
Oxidized adrenaline (mg.)	0	0	0·4	0·4
O_2 uptake (μl./10 min.)	55; 57	75; 77	20; 20	129; 132

When the ascorbic acid was added, without previous incubation, only a very slight cytochrome *c* stimulation was found under the conditions of Exps. 1 and 2. There seems little doubt (Fig. 2) that the leucoadrenochrome-adrenochrome system acts as a hydrogen carrier in the oxidation of adrenaline, and it is likely that hydrogen peroxide is formed in the reaction (see p. 372). Thus 'oxidized adrenaline' solutions may differ from mixtures of adrenaline and adrenochrome in containing small amounts of hydrogen peroxide. When Exps. 1 and 2 of Table 2 were repeated in the presence of 10^{-3}M-hydrogen peroxide, a similar cytochrome *c* stimulation was found without previous incubation.

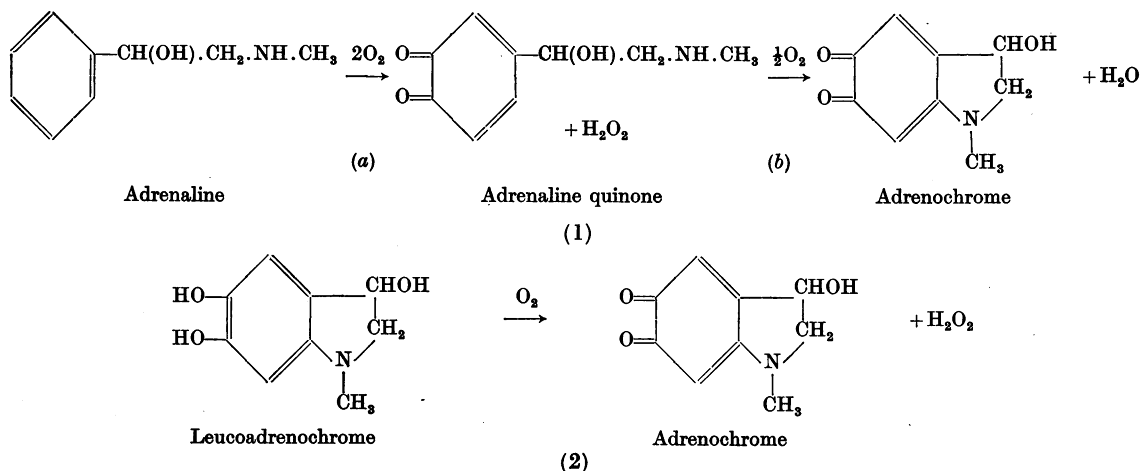
DISCUSSION

The oxidation of adrenaline

On shaking solutions of adrenaline in air at pH 7·0, a relatively slow, autocatalytic oxidation occurs, presumably involving reactions (1*a*) and (1*b*) (see p. 372).

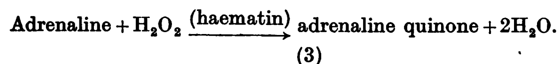
Melanin is eventually formed by unknown reactions, probably involving further oxidation and polymerization. The rate, followed by measurement

of oxygen uptake, is typical of an autocatalytic process (Fig. 1, curve *A*); it is possible that this is due to adrenochrome, as formed, acting as a hydrogen carrier, being reduced by adrenaline to leucoadrenochrome with reoxidation by oxygen (reaction (2)).



This mechanism is supported by the results shown in Fig. 2. Reaction (1*a*) is probably slow compared with (1*b*), and it is probably (1*a*) which is catalyzed by the adrenochrome (see below, and cf. also LuValle & Weissberger, 1947).

The stimulation by cytochrome *c* and methaemoglobin of the oxidation of adrenaline is clearly a peroxidative effect, since it is stimulated by hydrogen peroxide and inhibited by catalase. It is probably again reaction (1*a*) which is catalyzed, becoming now reaction (3).



$$\text{Adrenaline} + H_2O_2 \xrightarrow{\text{(haematin)}} \text{adrenaline quinone} + 2H_2O. \quad (3)$$

Autocatalysis here is probably due to two factors, adrenochrome as it is formed catalyzing the oxidation as a hydrogen carrier, and the hydrogen peroxide formed (reaction (2)) contributing to the peroxidative catalysis.

The postulation of adrenochrome as a hydrogen carrier in the oxidation of adrenaline may seem invalid, since it requires the reduction of the adrenochrome by the more electropositive adrenaline (Table 3).

However, Ball & Chen (1933) measured a thermodynamically reversible step in the oxidation of adrenaline in acid solution, but found that at pH 7 the oxidant of the system was extremely unstable. There is little doubt that they were dealing with the system adrenaline ⇌ adrenaline quinone (1*a*), the latter undergoing at pH 7 a very rapid irreversible change with ring closure to yield adrenochrome by oxidation (either directly or after rearrangement to leucoadrenochrome), or by dismutation.

Such a system with an unstable component may be completely oxidized by another much more electronegative system. Thus Ball & Chen (1933) found that at pH 7 one equivalent of adrenaline (E'_0, pH 7 = +0·388 V) was capable of reducing four equivalents of 2:6-dichlorophenolindophenol (E'_0, pH 7 = +0·217 V).

The oxidation of ascorbic acid by adrenochrome

In catalyzing the oxidation of ascorbic acid (Fig. 3) adrenochrome again acts as a hydrogen carrier. A similar oxidation of the still more electro-

Table 3. *Oxidation-reduction potentials*

Substance	E'_0 at pH 7·0 (V)	Temp. (°)	Reference	Method
Adrenaline	+0·388	30	Ball & Chen (1933)	Oxidative
Catechol	+0·360	30	Ball & Chen (1933)	Oxidative
Quinol	+0·271	30	Ball & Chen (1933)	Oxidative
Leucoadrenochrome	+0·044	20	Wiesner (1942)	Polarographic
Ascorbic acid	−0·066	35·5	Borsook & Keighley (1933)	Potentiometric (method of mixtures) extrapolated to pH 7·0
Cysteine	−0·390	25	Borsook, Ellis & Huffman (1937)	Thermal

negative cysteine might be expected. The oxidation of cysteine is very complex, however (cf. Remick, 1943; Borsook *et al.* 1937), and E_0' values at pH 7 as high as $+0{\cdot}06$ V have been recorded (Williams & Drissen, 1930).

It is possible that adrenaline may be oxidized in living tissues by non-enzymic mechanisms such as those described above. The importance of adrenochrome has been stressed by Veer (1942*a*), who brought forward evidence that it is biologically active, and that it is a melanin precursor *in vivo*. The closely related pigment hallachrome was found by Friedheim (1932, 1933) to increase the oxygen consumption of erythrocytes, and probably plays a role in the maturation of reticulocytes (Gad, Jacobsen & Plum, 1944). Veer (1942*b*) obtained evidence for somewhat similar action of adrenochrome, in that it augments the leucocyte migration of animal bone-marrow transplantations *in vitro*.

The catalysis by adrenochrome of adrenaline oxidation is analogous to the non-enzymic step in the oxidation of tyrosine discussed by Kertész (1948), in that in each case the catalyst is an *o*-quinone. Leucoadrenochrome, however, is very rapidly autoxidized at pH 7, while dihydroxyphenylalanine oxidation is rapid only when catalyzed enzymically.

SUMMARY

1. The oxidation of adrenaline by atmospheric oxygen at pH 7 is catalyzed peroxidatively by cytochrome *c* and by methaemoglobin. Traces of hydrogen peroxide originally formed by autoxidation of adrenaline are later augmented by autoxidation of leucoadrenochrome.

2. The oxidation of adrenaline is catalyzed by adrenochrome, which acts as a hydrogen carrier.

3. Adrenochrome acts as a hydrogen carrier in catalyzing the oxidation of ascorbic acid by atmospheric oxygen. It does not affect the rate of oxidation of cysteine, catechol or quinol at pH 7.

4. The catalysis of the oxidation of ascorbic acid by 'oxidized adrenaline' solutions appears to be due to the adrenochrome content of these solutions.

Dr R. Lemberg is thanked for his guidance throughout the work, and Dr A. Albert for his continued interest. This work was financed by grants from the Wellcome Foundation and the National Health and Medical Research Council of Australia.

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The Substrate Specificity of the Tyrosine Decarboxylase of *Streptococcus faecalis*

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(*Received* 6 *August* 1948)

Streptococcus faecalis has been shown to possess an enzyme which decarboxylates L-tyrosine and L-3:4-dihydroxyphenylalanine, with the formation of carbon dioxide and the corresponding amines; this enzyme is known as tyrosine decarboxylase (Gale, 1940; Epps, 1944). L-3:4-Dihydroxyphenylalanine is also a substrate of the DOPA decarboxylase of mammalian tissues. This enzyme attacks neither L-tyrosine nor L-phenylalanine (Blaschko, 1939), but it does decarboxylate L-2:5-dihydroxyphenylalanine, L-*m*-hydroxyphenylalanine and L-*o*-hydroxyphenylalanine (Blaschko & Sloane-Stanley, 1948; Blaschko, 1949). The experiments described in this paper were done in order to find out whether 2:5-dihydroxyphenylalanine, *m*-hydroxyphenylalanine and *o*-hydroxyphenylalanine are substrates of the streptococcal tyrosine decarboxylase.