LXXXIII. ADRENALINE AND ADRENOCHROME

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GREEN & BROSTEAUX [1936] and Green [1936, 2] showed that the lactic and malic dehydrogenases of animal tissues could react with oxygen only in presence of coenzyme I, a carrier and a ketone fixative. The dehydrogenase catalysed the transfer of hydrogen from the substrate to the coenzyme; in turn the reduced coenzyme reduced the carrier, and finally reduced carrier reacted with molecular oxygen. The function of the ketone fixative consisted in binding the keto-acid formed by the oxidation of either lactic or malic acid. The product of oxidation in both cases completely arrested the catalytic oxidation unless removed by the ketone reagent.

Adrenaline, flavin and flavoprotein were the only substances occurring in animal tissues which were found capable of acting as carriers in the lactic and malic systems. The mechanism of the flavin and flavoprotein effects was perfectly clear. These substances were alternately reduced by the coenzyme and oxidized by molecular oxygen. This simple interpretation however failed to account for the adrenaline effect. The experiments of Green & Brosteaux [1936] and Green [1936, 2] showed clearly that the action of adrenaline as an oxidation carrier involved a complicated mechanism. The present communication deals with the analysis of the adrenaline effect in the lactic and malic systems of animal tissues.

I. *Experimental details*

The lactic and malic dehydrogenases were prepared from the heart muscle of pig by the method of Green & Brosteaux [1936]. The preparation of coenzyme I is also described in that paper.

The manometric experiments were carried out in Barcroft differential manometers at 38°. The reactions were started after equilibration by dislodging the Keilin cups containing adrenaline into the main portion of the manometer cups. The rate of shaking was 150 oscillations per min.

The adrenaline solutions were prepared by dissolving the recrystallized base in water containing the theoretical amount of hydrochloric acid necessary for neutralization (final *p*H 5). These solutions were stable for weeks when kept at 0°.

II. *The dependence of the adrenaline effect on the activity of the lactic and malic systems*

A mixture of enzyme, coenzyme I, malate (or lactate) and cyanide at *p*H 8 failed to take up any oxygen. With addition of adrenaline a vigorous uptake ensued. This effect was observed only when the complete malic or lactic system was present (cf. Tables I and II). If the enzyme, coenzyme, substrate or fixative was omitted, adrenaline had no influence on the oxygen uptake.

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Table I. *Adrenaline effect in the malic system*

Enzyme (ml.)	1·0	1·0	1·0	1·0	0
Coenzyme (ml.)	1·0	1·0	1·0	0	1·0
2*M* HCN (ml.)	0·1	0	0·1	0·1	0·1
M malate (ml.)	0·2	0·2	0	0·2	0·2
M/20 *l*-adrenaline (ml.)	0·1	0·1	0·1	0·1	0·1
Water (ml.)	0·9	1·0	1·1	1·9	1·9
μl. O_2 in 10 min.	370	34	4	4	4

Table II. *Adrenaline effect in the lactic system*

Enzyme (ml.)	1·5	1·5	1·5
Coenzyme (ml.)	0·5	0	0·5
2*M* HCN (ml.)	0·1	0·1	0·1
M lactate (ml.)	0·2	0·2	0
M/20 adrenaline (ml.)	0·1	0·1	0·1
Water (ml.)	0·9	1·4	1·1
μl. O_2 in 30 min.	153	0	3

The dependence of the adrenaline effect on the active functioning of the malic system could be demonstrated in the following ways:

(1) The rate of oxidation of malate is determined within certain limits by the concentration of coenzyme. If a functional relation exists between the adrenaline effect and the activity of the malic system, the magnitude of the adrenaline effect should similarly depend upon the concentration of coenzyme (cf. Fig. 1).

(2) The rate of oxidation of malate or lactate falls off at low concentrations of substrate. The adrenaline effect should therefore depend upon the concentration of substrate within the limit of the saturating concentration. Furthermore, with a limited amount of malate available, the increased uptake induced by adrenaline should correspond with the amount of oxygen required for the oxidation of all the available substrate to the corresponding keto-acid (cf. Table III).

(3) If adrenaline is acting in the capacity of a carrier, the disappearance of malate and the appearance of oxaloacetate, the oxidation product, should be capable of demonstration. The estimation of *l*-malic acid

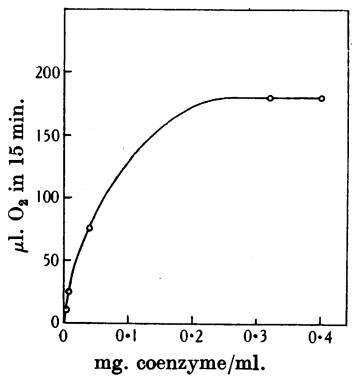


Fig. 1. Effect of the concentration of coenzyme on the magnitude of the adrenaline effect.

The manometers contained 1 ml. enzyme, 0·5 ml. *M*/2 glycine-phosphate buffer *p*H 8·5, 0·1 ml. 2*M* HCN, 0·1 ml. *M*/20 adrenaline and 0·2 ml. *M* malate.

Table III. *The dependence of the adrenaline effect on the concentration of malate and lactate*

	μl. O_2			
	10 min.	20 min.	30 min.	45 min.
System + 0·2 ml. *M* malate	348	481	510	—
System + 0·1 ml. *M*/10 malate	71	109	120	—
System + 0·1 ml. *M*/100 malate	0	0	0	—
System + no substrate	0	0	0	2
System + 0·2 ml. *M* lactate	—	—	252	338
System + 0·5 ml. 0·2*M* lactate	—	—	122	169
System + 0·1 ml. 0·2*M* lactate	—	—	22	42

The system contained 1 ml. enzyme, 0·5 ml. coenzyme, 0·1 ml. 2*M* HCN and 0·1 ml. *M*/20 *l*-adrenaline. The theoretical uptake for 0·1 ml. *M*/10 malate is 112 μl. O_2.

was carried out by the polarimetric method of Auerbach & Kruger [1923]. Malate was found to disappear in the presence of adrenaline and the complete system.

	mg. *l*-malic acid at end of 30 min.
Complete system	18·2
Control—no coenzyme	26·0
Control—no adrenaline	26·0

The complete system contained 2 ml. enzyme, 2 ml. coenzyme, 0·2 ml. 2 *M* HCN, 0·2 ml. *M*/10 adrenaline and 0·3 ml. *M* malate. The mixture was treated with 0·3 ml. glacial acetic acid and an equal volume of 5% uranium acetate. After filtration through kieselguhr, the filtrate was tested polarimetrically. The initial value of malate has no significance, since in presence of fumarase part of the added malate was converted into fumarate during the course of the experiment. A disappearance of 4·38 mg. *l*-malate per ml. enzyme corresponds to an oxygen uptake of 368 μl. O_2. The observed uptake was 420 μl. O_2. The isolation of oxaloacetic acid from the enzyme mixture containing cyanide has not yet been accomplished. Added oxaloacetic-cyanohydrin could not be recovered from the enzyme mixture and it seems very probable that the cyanohydrin undergoes decomposition in the course of the experiment.

(4) Pyruvate and oxaloacetate, which specifically inhibit the enzymic oxidations of lactate and malate respectively, should depress to a corresponding degree the effect of adrenaline. It is noteworthy that, to produce inhibition in presence of cyanide, pyruvate and oxaloacetate must be added in concentrations equivalent to that of cyanide, otherwise no appreciable concentration of inhibitor is available (cf. Table IV).

Table IV. *Effects of pyruvate and oxaloacetate on adrenaline effects*

	μl. O_2	
	10 min.	15 min.
Complete lactic system	—	90
+0·5 ml. *M*/5 pyruvate	—	0
Complete malic system	229	420
+0·3 ml. *M*/5 oxaloacetate	0	0

The complete systems contained 1 ml. enzyme, 0·6 ml. coenzyme, 0·1 ml. 2 *M* HCN, 0·1 ml. *M*/20 adrenaline and 0·2 ml. *M* substrate (lactate or malate).

(5) Lastly, if the action of adrenaline is catalytic, the rate of oxidation of malate should depend upon the concentration of adrenaline only below some saturation concentration. Above that level no further increase of the oxygen uptake should accompany an increase in the adrenaline effect. Green & Brosteaux [1936] and Green [1936, 2] have shown that the effect of the concentration of adrenaline on the rate of oxidation of lactate and malate conforms to theory. The concentration of adrenaline which permitted of half-speed oxidation was about 0·07 mg. In the present study, the smallest amount of adrenaline which could just produce a measurable effect in the reconstructed lactic or malic system was found to be 0·0006 mg. per ml. (cf. Fig. 2).

The results of these various tests uniformly support the hypothesis that adrenaline was acting as a respiration carrier in the lactic and malic systems

prepared from heart muscle of pig, and rule out the possibility that adrenaline was merely undergoing autoxidation under the conditions of the experiment.

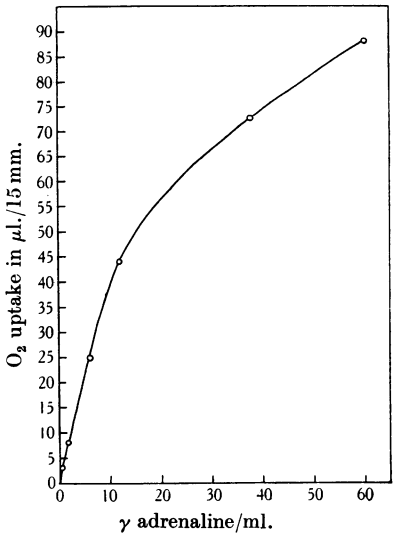


Fig. 2.

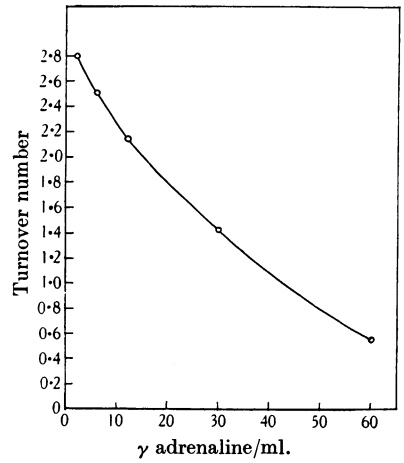


Fig. 3.

Fig. 2. Effect of adrenaline concentration on the rate of oxidation.
The manometers contained 1 ml. enzyme, 0·5 ml. coenzyme, 0·1 ml. 2*M* HCN, 0·1 ml. *M* malate, 0·5 ml. *M*/2 buffer *p*H 7·6 and 0·1 ml. *M*/20 H_2O_2. There was no uptake in 20 min. in the blank without adrenaline.

Fig. 3. The effect of the adrenaline concentration on the turnover number.
Details as for Fig. 2.

III. *The oxygen equivalence and the turnover number of adrenaline*

To satisfy the criterion of a respiration carrier, the total oxygen uptake induced by a given amount of adrenaline should be many times that required for the single reversible oxidation of adrenaline. In experiments designed to test this point, it was desirable to use a small amount of adrenaline and a relatively large excess of the enzyme system. Using 0·01 mg. of adrenaline in 3·3 ml. of the malic system, an oxygen uptake was observed in 30 min. which was 40 times that required to oxidize adrenaline to the corresponding quinone. In other words each molecule of adrenaline reacted with 40 atoms of oxygen. We shall see later why this oxygen equivalence number, high as it seems, is probably very much lower than the true equivalence number.

The turnover number has been defined as the number of times a molecule of carrier is reduced and oxidized in 1 min. The highest number observed *in vitro* is 300 for cytochrome *c* acting in the α-glycerophosphate dehydrogenase system of animal tissues [Green, 1936, 1]. Warburg & Christian [1933] found that flavoprotein was reduced and oxidized some 50 times per min. in the hexosemonophosphate system of yeast. More recently Theorell [1936] showed that at oxygen tensions such as obtain in the animal cell, the turnover number of flavoprotein is of the order of 1–2. These numbers are maximum and are obtained only with very small amounts of carrier in contact with large excess of the enzyme system.

The turnover number of adrenaline under as ideal conditions as could be arranged was never higher than 5. Although this number is much less than that

of cytochrome *c*, it compares favourably with that for flavoprotein and indeed represents high catalytic activity. Furthermore, there is good evidence as shown later for believing that the experimentally observed value is appreciably lower than the theoretically attainable value.

The experiments in which the turnover number was determined were carried out with less than 0·07 mg. of adrenaline per ml. in presence of excess of the enzyme system at *p*H 7·3–7·6. The oxygen uptake was not linear and readings were taken at 3-min. intervals. The turnover number therefore applies to the highest rate observed for a 3-min. period. Fig. 3 shows the dependence of the turnover number on the concentration of adrenaline. The maximum turnover number reached in this particular experiment was only 2·8.

The difference between *in vitro* and *in vivo* numbers is instructive. Haas [1934] found that cytochrome in intact yeast was reduced and oxidized 4000 times per min. in contrast to the 300 times for cytochrome *c* in the α-glycerophosphate system and 30 times for cytochrome *c* in the succinic system of animal tissues and the lactic system of yeast [Ogston & Green, 1935]. This discrepancy must be bound up with the question of the physical organization of the intact cell. In the reconstructed system *in vitro* we are dealing with reactions in homogeneous solution. The rate of reaction is determined by the probability of collision between enzyme and substrate, enzyme and coenzyme and finally carrier and oxygen. This degree of randomness imposes a low limit to the speed of the net reaction. There is good reason for supposing that *in vivo* reactions take place at orientated surfaces—randomness being thereby minimized. Instead of the various components of the catalytic system colliding with one another in unpredictable order, the physical organization in the cell is probably such as to impose direction upon the order of chemical events. There is therefore little cause for surprise when the catalytic activity of carriers is found to be much smaller *in vitro* than *in vivo*.

IV. *The role of the ketone fixative*

Green & Brosteaux [1936] and Green [1936, 2] found that fixatives other than cyanide could be used in reconstructing the lactic and malic dehydrogenases of animal tissues. Semicarbazide, hydrazine and hydroxylamine could bind keto-acids but not as efficiently as cyanide. It was not perfectly clear whether the relative inefficiency of these fixatives was due to a slow reaction with the keto-acid or to a toxic effect on the dehydrogenases. Whatever the correct explanation, the fact remained that in presence of cyanide the rate of oxidation of malate was several times that in presence of the other fixatives. The objection may therefore be raised that cyanide was playing a specific role in the adrenaline effect.

Green & Williamson [1937] have shown that cyanide in aqueous solution combines almost instantaneously with pyruvic or oxaloacetic acid to form the corresponding cyanohydrin. These cyanohydrins are resistant to hydrolysis at neutral reaction. It is only by treatment with dilute alkali or by hydrolysis in boiling acid that the original keto-acids can be regenerated. The evidence is therefore clear that cyanide is acting in the capacity of an efficient ketone fixative.

The following experiment shows that adrenaline can act as a carrier in presence of semicarbazide as fixative:

	μl. O_2	
	5 min.	10 min.
0·1 ml. 2*M* HCN as fixative	217	390
0·5 ml. *M*/2 semicarbazide as fixative	75	166
Semicarbazide control with no malate	17	59
Semicarbazide control with no adrenaline	8	13

All manometer cups contained 1 ml. enzyme, 1 ml. coenzyme, 0·1 ml. *M* malate, 0·1 ml. *M*/10 adrenaline and 0·8 ml. buffer *p*H 8·4 unless otherwise stated.

It is noteworthy that the magnitude of the adrenaline effect was much smaller than in the presence of cyanide, and furthermore that the controls without adrenaline and without malate were not negative as was the case when cyanide was used as the fixative. The control oxygen uptake in absence of malate does not signify the uncatalysed autoxidation of adrenaline. In a later section, evidence is presented that the indophenol oxidase can catalyse the oxidation of adrenaline. Cyanide inhibits the indophenol oxidase completely—hence the cyanide control without malate was negative. Hydrazine and semicarbazide do not completely inhibit the indophenol oxidase. The use of these reagents as fixatives always entails therefore some oxidation of adrenaline which is independent of the malic or lactic systems.

Dewan, Green and Leloir in unpublished experiments on the β-hydroxybutyric dehydrogenase have found that under certain conditions no carrier is required for the reaction of the dehydrogenase system with molecular oxygen although coenzyme I is the intermediary between oxygen and the substrate. They have interpreted this result to mean that reduced coenzyme I can react directly with oxygen. Strong cyanide inhibited this direct reaction with oxygen, whereas the other ketone reagents did not interfere. Similarly they found that the lactic and malic dehydrogenases, particularly in presence of hydrazine as fixative, did not require any added carrier for the reaction with molecular oxygen. It appears very probable therefore that cyanide quite apart from acting as a fixative prevents the direct oxidation of the reduced coenzyme. There is therefore no direct reaction between the reduced coenzyme and oxygen in presence of strong cyanide unless a suitable carrier is provided.

The question may be raised whether carriers are necessary *in vivo* for the lactic, malic and β-hydroxybutyric systems in view of the fact that reduced coenzyme is capable of direct reaction with oxygen. The evidence is not clear at the moment just how this oxidation of reduced coenzyme by molecular oxygen takes place, but it seems probable that the process is catalysed by some enzyme system. That would mean that in absence of this enzyme system, carriers are essential for the reaction of coenzyme dehydrogenases with oxygen. Conversely in presence of the enzyme system, carriers would be superfluous. It is fortunate therefore that cyanide, by suppressing the direct oxidation of reduced coenzyme, allows the carrier method of oxidation to be studied exclusively.

The difference in rate of oxidation of malate in presence of cyanide, compared with the rates of oxidation in presence of other fixatives, is not peculiar to the use of adrenaline as a carrier. The same difference occurs regardless of the nature of the carrier whether it be methylene blue, flavin or adrenaline.

V. *Specificity of adrenaline*

In all the experiments described above, *l*-adrenaline, the naturally occurring enantiomorph was used. A study of a series of adrenaline-like substances has shown that *d*-adrenaline, epinine ($(OH)_2C_6H_3CH_2CH_2NHCH_3$), corbasil ($(OH)_2C_6H_3CHOHCH(CH_3)NH_2$) and 3:4-dihydroxyphenylmethylaminopropanol can act in a similar way to *l*-adrenaline:

	μl. O_2 in 15 min.
Malic system + *l*-adrenaline	300
,, + *d*-adrenaline	300
,, + epinine	200
,, + corbasil	88
,, + 3:4-dihydroxyphenylmethylaminopropanol	540
,, no carrier	0

In each case 0·1 ml. $M/10$ carrier was added. The controls without malate were negative. Adrenalone, *d*- and *l*-*p*-sympatol, arterenol, "dopa", tyrosine, tyramine, catechol, ephedrine, protocatechuic acid, quinone, thyroxine and ω-aminoacetocatechol were inactive as carriers. There is no doubt therefore that the adrenaline effect is highly specific for a particular configuration and that we are not dealing with a general effect of catechol derivatives.

Although the malic and lactic systems showed no preference for either one of the two optical isomerides of adrenaline, the specificity was complete as far as the substrates were concerned. Whether *d*- or *l*-adrenaline were used, only *l*(+)lactate and *l*(−)malate could be oxidized. Their optical enantiomorphs were not oxidized.

VI. *The factors concerned in the adrenaline effect*

The reconstructed heart malic dehydrogenase system worked equally well with methylene blue or adrenaline as carrier. However, after treating the enzyme preparation with adsorbing agents the ratio $\frac{\text{rate of oxidation by methylene blue}}{\text{rate of oxidation with adrenaline}}$ did not remain constant. For example kaolin changed the ratio from the control value of 1·4 to 14·2. Kieselguhr had a similar though not so pronounced effect; charcoal had no appreciable effect (cf. Table V).

Table V. *Adrenaline effect with purified heart enzyme*

	μl. O_2 in 10 min.		
	Methylene blue	Adrenaline	Ratio $\frac{\text{methylene blue}}{\text{adrenaline}}$
Original enzyme	300	218	1·39
Kaolin-treated	94	6·6	14·2
Kieselguhr-treated	197	46	4·27
Charcoal-treated	266	318	0·89

Each manometer contained 1 ml. enzyme, 1 ml. coenzyme, 0·1 ml. $M/20$ adrenaline or 0·2 ml. 0·5% methylene blue, 0·2 ml. 2 M HCN and 0·2 ml. M malate.

These results indicate that some factor essential for the adrenaline effect is removed by adsorption on kaolin or kieselguhr. The conclusion also follows that, in addition to the dehydrogenase and coenzyme, some other factor is concerned in the mechanism by which adrenaline acts as a carrier.

The malic dehydrogenase can be prepared from practically every tissue of the animal body. If there is an additional factor for the adrenaline effect, we should expect that not all preparations of malic dehydrogenase would be capable of utilizing adrenaline as a carrier. A study of the malic dehydrogenases of rabbit tissues showed that only the heart preparation and to a slight extent the skeletal muscle preparation were positive for adrenaline. Preparations of brain, liver and kidney were negative, although they oxidized malate quite rapidly in presence of methylene blue (cf. Table VI).

Table VI. *The distribution of the adrenaline factor in rabbit tissues*

	μl. O_2 in 30 min.				
	Kidney	Brain	Liver	Muscle	Heart
System + malate + adrenaline	0	0	13·6	26·8	114
System + adrenaline	0	0	12·0	12·4	17
System + malate + methylene blue	173	70	133	246	104

The system contained in each case 1·5 ml. enzyme, 1·0 ml. coenzyme, 0·2 ml. 2 M HCN, 0·1 ml. $M/20$ adrenaline or 0·2 ml. 0·5% methylene blue. The malate where added was 0·2 ml. M.

The enzymes were prepared from the various tissues by the method of Green & Brosteaux [1936].

No systematic study has yet been made of the distribution of the adrenaline factor. The breast muscle of the pigeon was found to be a particularly rich source. The skeletal muscle of the pig was another tissue besides heart which was found to contain the factor.

There is a serious difficulty in the way of studying the distribution of the adrenaline factor. Suppose that this factor is more easily washed out of brain, liver and kidney cells than out of heart or skeletal muscle cells. Since the method of preparation in all cases involved thorough washing of the tissue brei prior to grinding and extracting, the possibility is open that in cases where the factor was missing in the final enzyme preparation, it had been lost in the course of the preparation.

VII. *The adrenaline effect with other dehydrogenase systems*

Thus far only the lactic and malic dehydrogenases of heart have been demonstrated to be capable of utilizing adrenaline as a carrier. The β-hydroxybutyric enzyme prepared from the same source showed a definite though weak adrenaline effect:

	μl. O_2 in 15 min.
Complete adrenaline system	100
No adrenaline	0
No β-hydroxybutyrate	0

The complete system contained 1·5 ml. heart enzyme, 1 ml. coenzyme, 0·2 ml. 2*M* HCN, 0·1 ml. *M*/20 adrenaline and 0·2 ml. *M* *dl*-β-hydroxybutyrate.

With the exception of these three coenzyme I dehydrogenase systems of animal tissues, no other systems have been found to be positive. The succinic dehydrogenase of heart, the α-glycerophosphate dehydrogenase of rabbit muscle and the lactic dehydrogenase of yeast were negative (cf. Table VII). These

Table VII. *The adrenaline effect with cytochrome dehydrogenase systems*

	μl. O_2		
	10 min.	20 min.	30 min.
Yeast lactic system + methylene blue	143	268	378
Yeast lactic system + adrenaline	0	0	0
Muscle α-glycerophosphate system + methylene blue	—	420	—
Muscle α-glycerophosphate system + adrenaline	—	0	—
Heart succinoxidase + methylene blue	90	—	—
Heart succinoxidase + adrenaline	3	—	—

The enzymes were prepared by the methods of Ogston & Green [1935] and Green [1936, 1]. All the three systems contained 1 ml. enzyme, 1 ml. *M*/2 buffer *p*H 8·0, 0·2 ml. 2*M* HCN and 0·2 ml. *M* substrate. Usual concentrations of carrier.

systems do not require a coenzyme and normally react with oxygen through the intermediation of cytochrome [Ogston & Green, 1935; Green, 1936, 1].

The tentative hypothesis concerning the components necessary for the adrenaline effect may be formulated as follows. Any coenzyme I dehydrogenase system can react with adrenaline, provided the special factor concerned in the adrenaline effect is present. Systems which do not require a coenzyme do not react with adrenaline even in presence of the factor. No evidence however is available whether coenzyme II systems behave like those of coenzyme I.

VIII. *Inhibitors of the adrenaline effect*

Ascorbic acid and glutathione inhibited completely the adrenaline effect in extremely small concentrations (cf. Tables VIII and IX). It is generally known that these two substances protect adrenaline from autoxidation and it is conceivable that the mechanism of the inhibitor action consists in protecting

Table VIII. *Effect of ascorbic acid on adrenaline effect*

	μl. O_2		
	5 min.	10 min.	15 min.
Complete system	141	330	447
+0·5 ml. *M*/20 ascorbic acid	3·3	12·9	24·4
+0·1 ml. *M*/20 ascorbic acid	0	0	3
+0·3 ml. *M*/200 ascorbic acid	4·6	10·7	12·3

The complete system contained 1 ml. enzyme, 0·3 ml. coenzyme, 0·5 ml. buffer *p*H 7·8, 0·2 ml. 2*M* HCN, 0·1 ml. *M*/20 adrenaline and 0·2 ml. *M* malate. Total volume 3·3 ml.
The small oxygen uptake observed with the highest concentration of ascorbic acid used was shown to be due to slow autoxidation of ascorbic acid under these conditions.

Table IX. *Effect of reduced glutathione*

	μl. O_2 in 10 min.
Complete system	328
+0·5 ml. *M*/20 glutathione	23
+0·1 ml. *M*/20 glutathione	11
+0·2 ml. *M*/200 glutathione	338

Details as for Table VIII.

adrenaline against the catalytic oxidation. When adrenaline is replaced by adrenochrome, the actual reversibly reducible and oxidizable derivative of adrenaline, then ascorbic acid and glutathione have no inhibitory action.

IX. *The effect of* p*H*

The adrenaline effect with the malic system depended in an extraordinary way upon the *p*H (cf. Fig. 4). At *p*H 9 the effect was observed at once. With decrease of *p*H a lag period intervened before oxidation of malate started, the length of the lag period increasing until at *p*H 7·0 or below the adrenaline effect disappeared. Once the lag period was overcome, the rates of oxidation at the various *p*H values were not greatly different.

The *p*H curve could not be extended beyond 9, since the non-enzymic autoxidation of adrenaline came into play in the more alkaline range. Within the range of *p*H studied, the control uptakes without malate were very small or negative.

Different preparations of the malic dehydrogenase from pig's heart did not always correspond in the *p*H at which the adrenaline effect was negative. In some cases *p*H 7·3 was the limiting value; in others *p*H 6·8. Similarly there was variability in the lengths of the lag period from one preparation to another. But as will be seen later these variations can probably be explained in terms of differences in the amount of adrenaline factor and inhibitors present.

The dependence of the adrenaline effect on the *p*H and the existence of a lag period at neutral reaction have proved to be valuable assets for analysing the mechanism by which adrenaline acts catalytically. Clearly a factor which is essential to the adrenaline effect was either non-operative or inhibited at the *p*H values at which the lag periods were observed. That is to say the additional factor was

effective at *p*H 8 and above and progressively lost in efficiency with decreasing *p*H. The probable nature of the factor could therefore be arrived at by finding out what substances could eliminate the lag period.

The possibility of a haematin catalysis suggested trying cytochrome *c*. At *p*H 7·6 a mixture of malic dehydrogenase, cyanide, coenzyme I, malate and adrenaline failed to take up any oxygen in 20 min. With addition of 0·2 ml. *M*/1000 heart cytochrome *c* the reaction practically went to completion in that

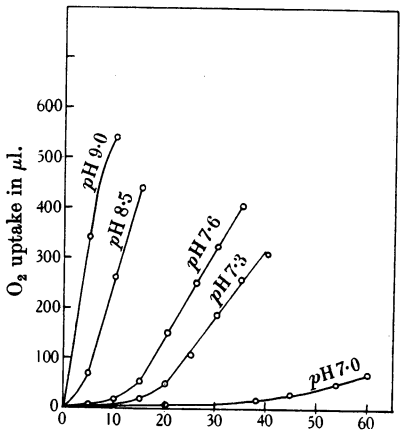


Fig. 4.

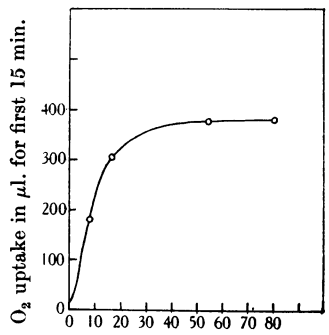


Fig. 5.

Fig. 4. The effect of *p*H on the lag period of the adrenaline effect.

The *p*H of the buffer solutions were determined electrometrically at 38°. The manometers contained 1 ml. enzyme, 1 ml. coenzyme, 1 ml. *M*/2 phosphate-glycine buffer, 0·1 ml. 2 *M* HCN, 0·1 ml. *M* malate and 0·1 ml. *M*/20 adrenaline.

Fig. 5. The effect of heart cytochrome *c* on the adrenaline effect at *p*H 7·6.

The manometers contained 1 ml. enzyme, 1 ml. coenzyme, 0·2 ml. 2 *M* HCN, 0·1 ml. *M*/20 adrenaline and 0·5 ml. *M*/2 buffer *p*H 7·6.

Table X. *Effect of heart cytochrome* c *on the lag period at* p*H 7·6*

	µl. O_2 in 20 min.
Adrenaline-malate system	0
+0·2 ml. *M*/1000 cytochrome *c*	260
Cytochrome *c* control—no malate	0
Cytochrome *c* control—no coenzyme	4

Details as in Table VIII.

time (cf. Table X). Cytochrome *c* had no effect on the oxygen uptake unless the complete system was present. Green [1936, 2] has demonstrated that cytochrome *c* alone cannot act as a carrier in the malic system.

The effect of the cytochrome concentration on the elimination of the lag period and on the rate of oxidation is shown in Fig. 5. Increase of cytochrome concentration did not affect the final rate but only the speed with which the reaction started. That is to say with or without cytochrome, once the reaction was under way the velocities were identical. Cytochrome was therefore acting in a trigger capacity.

Hydrogen peroxide behaved very much as cytochrome *c* (cf. Table XI). It eliminated the initial lag but did not influence the limiting velocity once that was

Table XI. *Effect of H_2O_2 on adrenaline lag period at* p*H 7·6*

	μl. O_2		
	10 min.	20 min.	35 min.
Adrenaline-malate system	5·5	17·7	163
+0·1 ml. *M*/10 H_2O_2	110	290	433
H_2O_2 control—no malate	0	0	0

Details as in Table VIII.

attained. Here again the effect of H_2O_2 was connected with the malic dehydrogenase-adrenaline system, for in absence of any one of the components hydrogen peroxide did not induce any oxygen uptake. Fig. 6 shows the effect of varying the concentration of hydrogen peroxide.

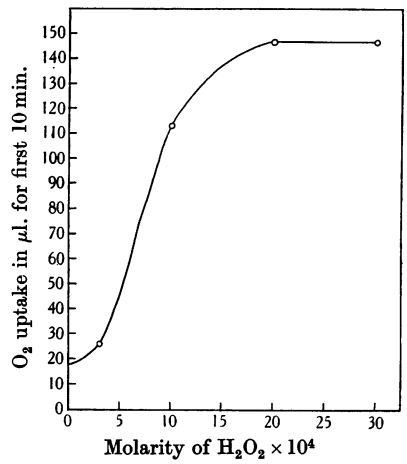


Fig. 6. The effect of hydrogen peroxide on the adrenaline effect at *p*H 7·6. Details as for Fig. 5.

At first glance it is difficult to understand why cytochrome *c* and H_2O_2 should produce the same effect. Adrenaline can be oxidized directly by cytochrome *c*. H_2O_2 however does not oxidize adrenaline at neutral or slightly alkaline reaction.

The amount of cytochrome *c* added to eliminate the lag period was sufficient to oxidize only an insignificant fraction of the total adrenaline present. Apparently then cytochrome *c* set the reaction off by reacting directly with adrenaline and thereby forming some of the adrenaline oxidation product. That is to say the lag period represents the delay in forming a sufficient quantity of the adrenaline oxidation product which is essential for the initiation of the reaction. If this hypothesis is correct we should expect that cytochrome *c* in the reduced form would have no effect on the lag period, since there would be no possibility of reaction with adrenaline. Such was indeed the case:

	μl. O_2	
	10 min.	20 min.
Adrenaline-malate system	0	75
+0·2 ml. *M*/1000 oxidized cytochrome *c*	166	354
+0·2 ml. *M*/1000 reduced cytochrome *c*	5	27

Now how can the H_2O_2 effect be brought into line with that of cytochrome *c*? In absence of any enzyme a large amount of adrenaline in contact with a small amount of cytochrome *c* will interact until all the cytochrome is reduced. The reaction then stops. If H_2O_2 is added to the system the oxidation of adrenaline continues until all the H_2O_2 is used up. The explanation is found in the two facts (1) that reduced cytochrome *c* is non-autoxidizable and (2) that reduced cytochrome *c* can be oxidized by H_2O_2. The chain of reactions is the following:

adrenaline + oxidized cytochrome *c* → oxidation product of adrenaline + reduced cytochrome *c*

reduced cytochrome *c* + H_2O_2 → oxidized cytochrome *c*.

The above model system consisting of adrenaline, cytochrome *c* and H_2O_2 has been studied manometrically as well as spectroscopically with the same results (cf. Table XII). The observed oxygen uptake is probably due mainly to the oxidation of leuco-adrenochrome by molecular oxygen.

Table XII. *Construction of model system for oxidation of adrenaline*

M/20 adrenaline (ml.)	0·3	0·3	0·3	0
M/1000 oxidized cytochrome *c* (ml.)	0·1	0	0·1	0·1
M/10 H_2O_2 (ml.)	0	0·1	0·1	0·1
M/2 buffer *p*H 7·0 (ml.)	2·0	2·0	2·0	2·0
2*M* HCN (ml.)	0·1	0·1	0·1	0·1
Water (ml.)	0·9	0·9	0·8	1·1
μl. O_2 in 20 min.	0	0	60	0

The suggestion is clear that the adrenaline factor, if not one of the cytochromes, is something in the nature of a haematin compound. The addition of H_2O_2 leads to the oxidation of adrenaline by the adrenaline factor in the malate system for the same reasons as apply to the model system studied above.

If the essential condition for eliminating the lag period is the production of a small quantity of the adrenaline oxidation product, we should expect that the oxidation product would itself be the most efficient reagent. This prediction was in fact realized. When adrenaline was replaced by adrenochrome the reaction showed no lag period and furthermore cytochrome *c* had no influence on the rate of oxidation (cf. Table XIII).

There are several lines of evidence which point to the adrenaline factor being a haematin and probably one of the cytochrome components. (1) The malic system of pig brain as normally prepared failed to show an adrenaline effect. With addition of cytochrome *c* the effect was observed. (2) The enzyme solution prepared from heart contains cytochrome *a*, *b* and *c* in definite though small amounts. Purification by adsorption on kieselguhr or kaolin leads to removal of the cytochrome as well as to removal of the adrenaline factor. (3) The cytochrome effect in eliminating the lag period applies not only to *l*-adrenaline but also to the other adrenaline-like substances which are active with the malic system. It must be more than a coincidence that cytochrome *c* can oxidize rapidly precisely those substances which give a positive adrenaline effect.

X. *Oxidation products of adrenaline*

In the complete adrenaline-malic or lactic system the end of the lag period and the commencement of rapid oxygen uptake was characterized by the appearance of a red colour in the solution. This red colour disappeared if the shaking was discontinued but reappeared on shaking with air and persisted as long as the solution was kept well oxygenated.

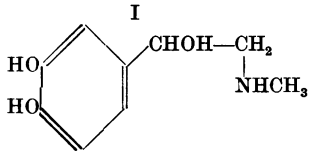
Adrenaline has been shown to act as a carrier in the coupled oxidation of substrates with catechol oxidase [Richter, 1934]. In this sytem the adrenaline is oxidized to a red coloured quinone which can then take part in secondary oxidations. The red colour observed in the adrenaline-malic system suggested that a similar mechanism might also operate here. Attempts were therefore made to prepare the red quinone by chemical means in order to test whether it could replace adrenaline in the malic system.

That red coloured oxidation products are formed from adrenaline by the action of oxidizing agents such as iodine, bromine or potassium iodate, or by autoxidation, is well known [Fränkel & Allers, 1909; Kisch, 1930]. A number of these products have been used for the colorimetric estimation of adrenaline; but

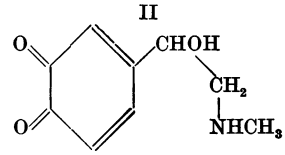
little is known of their chemical nature as, except for a product described by Richter & Blaschko [1937], they have not hitherto been isolated.

Ball & Chen [1933] measured the oxidation potentials of the system formed by treating adrenaline (I) with oxidizing agents and obtained evidence that the primary product formed is generally the corresponding ortho-quinone (II); but

I



II



this is an extremely unstable substance and at *p*H 7 it undergoes an irreversible change within 1/30 sec. to give a product of undetermined constitution.

XI. *Oxidation with chemical reagents*

Iodine. In the oxidation of adrenaline with iodine solution the end of the reaction, as shown by the attainment of a maximum colour intensity of the red product, was not reached until the equivalent of 3 or more atoms of oxygen per molecule of adrenaline had been added. The use of a limited amount of the oxidizing agent led to the formation of a mixture of products and unchanged adrenaline.

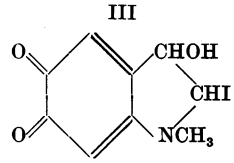
0·5 ml. *M*/20 adrenaline hydrochloride, 1 ml. 0·3*M* HCl and 1·2 ml. 10% sodium acetate were treated with 0·25–3·0 ml. *N*/10 iodine solution (total volume 6 ml.). After standing for 15 min. the excess of iodine was removed by extracting with ether and the concentration of red product estimated colorimetrically. It decomposed rapidly on treating with dilute hydrochloric acid or ammonia or on boiling. The colour faded on standing at 20° for a few hours. The red product was also formed when iodine was added to an alcoholic adrenaline acetate solution, but it decomposed when the alcohol was evaporated under reduced pressure.

Potassium ferricyanide. A red quinonoid product was formed by treating adrenaline with potassium ferricyanide. The rate of the reaction was increased by adding lead acetate to remove the ferrocyanide formed and so maintain the potential of the system.

A solution containing 0·2 ml. *M*/20 adrenaline hydrochloride, 0·5 ml. 2*M* sodium acetate buffer *p*H 4·6 and 0·3 ml. 0·67*M* lead acetate was treated with 0·3–2 ml. of *N*/10 potassium ferricyanide. The equivalent of 3 atoms of oxygen was required to produce a maximum colour intensity in 60 min. The colour faded on standing. Attempts to isolate the reaction product were unsuccessful.

Bromine. Richter & Blaschko [1937] have described a crystalline product containing iodine which was obtained by oxidizing adrenaline with potassium iodate. It had the properties of a dihydroindole derivative.

III



A closely similar product containing a bromine atom instead of the iodine atom has now been obtained by the action of bromine on adrenaline. Its solution in

water, in which it was sparingly soluble, was purple-red in colour and it crystallized from water in prisms with green metallic lustre. It decomposed on treating with dilute hydrochloric acid or ammonia or on boiling in aqueous solution. It had the properties of an *ortho*-quinone, since it was readily reduced by sulphur dioxide to a substance which gave the characteristic catechol reaction with ferric chloride. The substance had no basic properties, which makes it probable that, like the iodo-compound, it is also a dihydroindole derivative.

1 g. adrenaline was dissolved in 50 ml. 2 % acetic acid and 5 g. sodium acetate and 0·57 ml. bromine were added with vigorous shaking. The solution became deep red in colour and on standing in the ice chest crystals of the bromo-derivative separated. Yield 0·23 g. (Found (Weiler): C, 41·6; H, 3·5; N, 5·5; Br, 30·2 %. $C_9H_{10}O_3NBr$ requires C, 41·5; H, 3·9; N, 5·4; Br, 30·7 %.)

XII. *Oxidation with catechol oxidase*

Catechol oxidase at *p*H 7·3 catalysed the oxidation of adrenaline, but at this *p*H the red coloured product rapidly decomposed to give a black melanin-like substance. At *p*H 5 the red compound was much more stable, but with ordinary preparations of the catechol oxidase of potatoes the rate of oxidation at *p*H 5 was so much reduced that attempts to isolate the red product were unsuccessful.

Using a highly active preparation of the catechol oxidase of the mushroom *Agaricus campestris* kindly given by Prof. Keilin it was found possible to obtain a sufficiently rapid oxidation at *p*H 5 for a comparatively pure solution of the red quinone to be obtained. On rapid evaporation of this solution in a Fuchs's apparatus [Fuchs, 1928] crystals of the red compound were obtained.

A solution containing 0·5 g. adrenaline in 50 ml. was brought to *p*H 5 with acetic acid. Catechol oxidase was added and a rapid stream of oxygen was passed in through a sintered glass bubbler. The rate of oxygen uptake of a sample of the solution was estimated from time to time in a Barcroft apparatus. When the oxidation came to an end (2–3 hours at 20°) the solution was filtered and evaporated.

Subsequent work showed that this substance is probably identical with the red compound derived from adrenaline which operates in the malic system, as it behaved equally well as a carrier in the malic system and gave quantitatively the same turnover number. Investigation of the chemical constitution showed that it has the properties of *N*-methyl-2:3-dihydro-3-hydroxyindole-5:6-quinone. The shorter name adrenochrome is proposed for this substance.

XIII. *Properties of adrenochrome*

Adrenochrome is a very unstable substance: it is completely destroyed in 4 min. by 1 % hydrochloric acid, and in 40 sec. by 1 % ammonia. At *p*H 7·3 and 37° the red colour disappears in 35 min., and even under the optimum conditions for keeping the solution, at *p*H 4·0 and 0°, marked decomposition and melanin formation could be observed in 4–5 hours. In the dry crystalline condition adrenochrome could be kept for several weeks, but on standing in a vacuum desiccator it decomposed to give a brown amorphous product. The crystalline substance appears to be a hydrate. The crystals decompose on heating at about 115–120°.

Adrenochrome is very soluble in water and crystallizes from water only on evaporation practically to dryness. It is also readily soluble in methyl alcohol. It is removed quantitatively from the aqueous solution by adsorption on charcoal, and can be eluted again from the charcoal with methyl alcohol.

XIV. *Constitution of adrenochrome*

In the oxidation of adrenaline catalysed by catechol oxidase the reaction came to an end when approximately 2 atoms of oxygen per molecule of adrenaline had been taken up.

	μl. O_2			
	5 min.	10 min.	35 min.	Theoretical for 2 atoms oxygen
Enzyme alone	5	8	16	0
0·1 ml. *M*/20 adrenaline	88	94	110	112
0·2 ml.	156	185	213	224

The complete system contained 1 ml. enzyme solution, 1 ml. *M*/10 buffer *p*H 5·3 and adrenaline. Total volume 3·3 ml.

This would indicate the formula $C_9H_9O_3N$ for adrenochrome according to the equation:

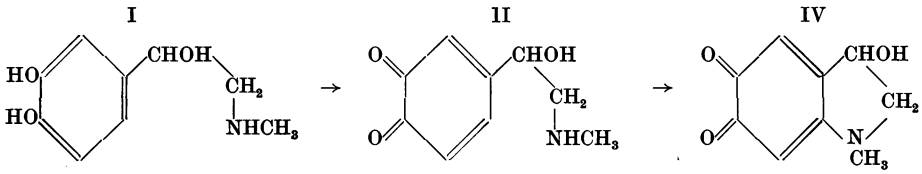
$$C_9H_{13}O_3N + O_2 \rightarrow C_9H_9O_3N + 2H_2O$$

This formula was confirmed by analysis (Weiler). (Found C, 57·5%; H, 6·0%; N, 7·2%. $C_9H_9O_3N$, $\frac{1}{2}H_2O$ requires C, 57·4%; H, 5·4%; N, 7·4%).

Adrenochrome behaves as an *ortho*-quinone in that it is readily reduced by sulphur dioxide, hydrogen sulphide or palladium and hydrogen to give a colourless leuco-compound which give the colour reactions of a catechol derivatives with ferric chloride. Leuco-adrenochrome is optically active and has $[\alpha]_D^{18°} = 79·2°$. The asymmetric CH.OH group is therefore probably intact in adrenochrome. Adrenochrome is much less basic than adrenaline since it crystallizes from dilute acetic acid in the free condition and not as a salt.

The aqueous solutions of all the simple *ortho*-quinones which are known are orange or yellow in colour. The brilliant red colour of adrenochrome indicated that substitution in the benzene nucleus might have occurred; the colour is suggestive of the aminoquinones which are also red. The ultraviolet absorption spectrum of leuco-adrenochrome (2500–4000 Å.) also differs widely from that of adrenaline. Some change in the groups attached to the benzene nucleus, which determines the absorption in this range of wave-lengths, is again indicated.

Adrenochrome contains 4 hydrogen atoms less than adrenaline. Two of these hydrogen atoms have been removed in quinone formation: the other two hydrogen atoms have been removed in a reaction which has diminished the basicity of the nitrogen atom and at the same time appears to have effected a substitution in the benzene nucleus. This is best explained by ring closure between the nitrogen atom and the benzene nucleus to give a dihydroindole derivative IV.

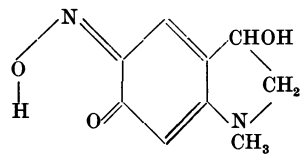


It is probable both from the known mode of action of catechol oxidase and from Ball & Chen's work that the first stage in the oxidation of adrenaline to adrenochrome is the formation of the *ortho*-quinone (II). The work of Raper [1927] on the formation of indole derivatives from dihydroxyphenylalanine gives an example of a similar reaction occurring in an analogous series of compounds. The proposed

constitutional formula for adrenochrome gives an interpretation of all the properties hitherto observed and indicates that it may be related to the naturally occurring pigment hallachrome which was isolated by Mazza & Stolfi [1931] from the polychaete *Halla parthenopea*.

On treatment with hydroxylamine adrenochrome gave a quinoneoxime (V)

V



which crystallized from water in bright orange needles. The oxime was a much more stable substance than adrenochrome and melted sharply at 278°. (Found C, 48·8, 48·8; H, 5·9, 5·8; N, 12·4, 12·7 %. $C_9H_{10}O_3N_2$, $1\frac{1}{2}H_2O$ requires C, 48·8; H, 5·9; N, 12·7 %.) When treated with a mixture of potassium iodate and iodide, adrenochrome gave a product identical with the iodo-compound described in section XI. This confirms the constitution which has been given to adrenochrome, since the constitution of the iodo-compound has been determined independently.

XV. *Oxidation of adrenaline by haematin derivatives*

Adrenaline was found to be oxidized by a number of haematin derivatives, including methaemoglobin and the cytochromes.

Haematin. 0·5 ml. 1 % adrenaline hydrochloride and 1 ml. *M*/1·5 phosphate buffer *p*H 7·5 were added to a solution containing 0·5 mg. of the disodium salt of haematin in 1 ml. of water. On adding a few drops of pyridine and allowing the mixture to stand at 20° for a few minutes, crystals of pyridine haemochromogen separated out. They were identified by their characteristic crystalline habit and absorption bands at 5580 and 5260 Å.

Methaemoglobin. A solution of methaemoglobin was prepared by oxidizing 5 ml. human blood diluted 1:20 with 2 mg. potassium ferricyanide and dialysing to remove the excess of the reagent. On adding 1 ml. *M*/1·5 buffer *p*H 7·3 and 0·5 ml. 1 % adrenaline hydrochloride to 1 ml. of the solution and incubating in a vacuum tube at 37° the methaemoglobin band at 6330 Å. slowly disappeared. In 30 min. it had been replaced by the haemoglobin spectrum. The solution then showed the bands of oxyhaemoglobin on shaking in the air.

Cytochromes a, b *and* c. Cytochrome *c* is reduced very rapidly by adrenaline at *p*H 7·3. Cytochrome *a* is reduced less rapidly, while with cytochrome *b* the reduction was very slow. The reduction of cytochrome *a* and *b* was faster at more alkaline reactions.

A solution containing 0·1 ml. of 0·1 % cytochrome *c* and 1 ml. *M*/10 phosphate buffer *p*H 7·3 reacts almost instantaneously with 0·5 ml. 1 % adrenaline hydrochloride solution at 20° giving the absorption bands of reduced cytochrome. Vacuum tubes containing 2·5 ml. cytochrome *a* and *b* preparation from pig's heart, 0·5 ml. *M*/1·5 phosphate buffer *p*H 7·3 and 0·5 ml. 1 % adrenaline hydrochloride in 5 ml. show distinct bands of reduced cytochrome *a* after incubating at 30° for 45 min. Under the same conditions cytochrome *b* showed practically no reduction in this time, but at *p*H 8·8 a distinct reduction of the *b* component could be observed.

XVI. *Oxidation by indophenol oxidase-cytochrome system*

A preparation of indophenol oxidase obtained from pig's heart muscle by the method of Ogston & Green [1935] brings about a rapid oxidation of adrenaline in the presence of cytochrome *c*.

	μl. O_2 in 5 min.
Enzyme + adrenaline	14
Enzyme + cytochrome *c* + adrenaline	189
Boiled enzyme + adrenaline	0
Boiled enzyme + cytochrome *c* + adrenaline	0

In each tube 1 ml. enzyme, 0·2 ml. *M*/10 adrenaline and 1 ml. *M*/2 phosphate buffer *p*H 7·6 containing *M*/2 glycine. The enzyme was not entirely free from cytochrome and hence the enzyme and adrenaline showed a certain oxygen uptake without added cytochrome. The tubes with cytochrome *c* contained 0·3 ml. *M*/1000. Total volume 3·3 ml.

The dependence of the rate of oxidation on the *p*H was shown by the following experiments carried out under similar conditions but with different buffer solutions

	μl. O_2 in 5 min.		
*p*H ...	7·0	7·6	8·4
Enzyme + adrenaline	6	14	73
Enzyme + adrenaline + cytochrome *c*	129	189	290

The buffer solutions used were made up with *M*/2 glycine which has been shown by Wiltshire [1931] to inhibit the spontaneous autoxidation of adrenaline. The system was completely inhibited by *M*/1000 cyanide.

Besides adrenaline a number of other catechol derivatives and sympathomimetic drugs have been tested and a marked specificity of action was observed. It might have been anticipated on structural grounds that adrenalone and sympatol would be less readily oxidized, but the low rate of oxidation of dihydroxyphenylalanine was unexpected, as also was the specificity shown in the different rates of oxidation of *d*- and *l*-adrenaline. A further point of interest is the different speeds of oxidation of colephrine and corbasil, which differ only in the presence or absence of a methyl group on the nitrogen atom.

		μl. O_2 in 5 min.	
		Without cytochrome	With cytochrome
l-Adrenaline	$(OH)_2C_6H_3.CHOH.CH_2.NHCH_3$	4	246
d-Adrenaline	$(OH)_2C_6H_3.CHOH.CH_2.NHCH_3$	2	155
Epinine	$(OH)_2C_6H_3.CH_2.CH_2.NHCH_3$	14	238
dl-Dopa	$(OH)_2C_6H_3.CH_2.CH(COOH)NH_2$	0	1
ω-Aminoacetocatechol	$(OH)_2C_6H_3.CO.CH_2.NH_2$	0	30
Adrenalone	$(OH)_2C_6H_3.CO.CH_2.NHCH_3$	6	22
dl-Arterenol	$(OH)_2C_6H_3.CHOH.CH_2.NH_2$	0	174
l-m-Sympatol	$(OH)C_6H_4.CHOH.CH_2.NHCH_3$	0	0
dl-Corbasil	$(OH)_2C_6H_3.CHOH.CH(CH_3).NH_2$	2	246
dl-Colephrine	$(OH)_2C_6H_3.CHOH.CH(CH_3).NHCH_3$	2	414

Each tube contained 0·7 ml. enzyme, 1 ml. phosphate-glycine buffer *p*H 7·6, 0·3 ml. *M*/1000, cytochrome *c* and 0·3 ml. *M*/10 adrenaline derivative.

XVII. *Action of adrenochrome in the malic system*

Adrenochrome acted in the same way as adrenaline as a carrier in the malic system, the only difference between the two being that with adrenochrome there was no lag period; the rapid oxygen uptake began immediately. The two compounds even showed the same turnover number (T.N.) at equivalent concentra-

Table XIII. *Adrenaline and adrenochrome at* p*H 7·6*

	μl. O_2 in 20 min.
System + adrenaline	0
System + adrenaline + cytochrome *c*	260
System + adrenochrome	274
System + adrenochrome + cytochrome *c*	290

The system contained 1 ml. enzyme, 1 ml. coenzyme, 0·5 ml. *M*/2 buffer *p*H 7·6, 0·1 ml. 2 *M* HCN and 0·2 ml. *M* malate.

tions. There is therefore good reason for concluding that adrenochrome and the red oxidation product formed from adrenaline in the malic system are identical.

		μl. O_2 in 3 min.		
Adrenochrome	ml.	With malate	Without malate	T.N.
2·5 mg./ml.	0·5	80	12	0·5
,,	0·1	45	5	1·4
0·25 mg./ml.	0·3	16	0	1·9
,,	0·1	15·5	0	5·3

The complete system contained 1 ml. enzyme, 1 ml. coenzyme, 0·1 ml. *M* malate, 0·1 ml. 2 *M* HCN and 0·8 ml. *M*/2 phosphate-glycine buffer *p*H 7·6. Total volume 3·3 ml.

Adrenochrome, when added in very small amounts to the complete adrenaline-malic system, had the effect of terminating the lag period and accelerating the initiation of the reaction:

	μl. O_2	
	5 min.	10 min.
Adrenaline-malic system	0	0
Adrenaline-malic system + adrenochrome	39	97
Malic system + adrenochrome	7	19

The amount of adrenochrome added was 0·5 ml. 0·25 mg./ml. solution. The complete system contained 1·3 ml. enzyme, 1 ml. coenzyme, 0·8 ml. *M*/2 glycine, phosphate buffer *p*H 7·6, 0·2 ml. *M*/1 HCN, 0·1 ml. *M*/1 malate and 0·1 ml. *M*/10 adrenaline.

XVIII. *Discussion*

It has been shown that malic acid is rapidly oxidized by the system comprising coenzyme, cyanide, adrenaline and the malic dehydrogenase of heart muscle. A general scheme for the reactions involved is given in Table XIV.

The initiation of the reaction depended on the primary oxidation of the adrenaline to a red pigment, adrenochrome (reactions I and II). Addition of oxidizing agents such as cytochrome *c* or hydrogen peroxide hastened this oxidation; conversely reducing agents such as glutathione or ascorbic acid inhibited the oxidation.

When once the adrenochrome had been formed the oxidation of malate proceeded by reactions III, IV and V. Adrenochrome was reduced by the malic system to leuco-adrenochrome which in turn reoxidized, so that the adrenochrome acted as an oxygen carrier.

In considering how the adrenaline became oxidized to adrenochrome in the first place, it was found that under the conditions of the experiment, i.e. in the presence of cyanide, glycine and the enzyme, adrenaline did not autoxidize. The factor responsible for the oxidation of adrenaline to adrenochrome was probably a haematin compound similar to cytochrome which could be seen spectroscopically to be present in the enzyme preparation and which has been shown to oxidize adrenaline.

Table XIV

I	O_2	+	Reduced cytochrome	→	Oxidized cytochrome	+	H_2O	
II	Oxidized cytochrome	+	Adrenaline	→	Adrenochrome	+	Reduced cytochrome	
III	O_2	+	Leuco-adrenochrome	→	Adrenochrome	+	H_2O_2	
IV	Adrenochrome	+	Reduced coenzyme	→	Oxidized coenzyme	+	Leuco-adrenochrome	
V	Oxidized coenzyme	+	Malate	→	Oxaloacetate	+	Reduced coenzyme	
VI	Oxaloacetate	+	HCN	→	Cyanohydrin			

In the complete adrenaline-malic system the initiation of the reaction was greatly accelerated by the addition of a small trace of adrenochrome which produced an effect out of all proportion to that which would be expected from the actual amount of adrenochrome added. This trigger action of adrenochrome could be explained if the oxidation of each molecule of leuco-adrenochrome caused the production of a molecule of hydrogen peroxide (reaction III). The hydrogen peroxide so produced would oxidize a further quantity of adrenaline and so make a further amount of adrenochrome available as a carrier. The autocatalytic oxygen uptake curves that were regularly obtained with the adrenaline-malic system are also explained if hydrogen peroxide is produced in this reaction. In the absence of malate, added adrenochrome did not bring about the oxidation of adrenaline to adrenochrome: but this was to be expected, since only in the presence of malate would the turnover of adrenochrome be sufficiently rapid to liberate a significant amount of hydrogen peroxide in the solution.

In absence of cyanide, adrenaline and leuco-adrenochrome were readily oxidized by the indophenol oxidase-cytochrome system. The rapid oxygen uptake obtained with adrenochrome and the malic system in the presence of cyanide showed that for the operation of this system the indophenol oxidase was not necessary. Adrenochrome appeared in fact to provide a short cut for the oxidation of malic acid which could proceed by reactions III, IV and V without involving the indophenol oxidase system. Whether the oxidation of leuco-adrenochrome to adrenochrome was an autoxidation or whether it was enzymic remains to be investigated.

The observed value of 5 for the turnover number shows that adrenochrome is highly active as an oxygen carrier for the malic dehydrogenase system. This value was obtained despite the fact that adrenochrome is a very unstable substance and was in fact decomposing during the time that the measurement was made. The true catalytic activity of adrenochrome may therefore be even higher than this value for the turnover number would suggest.

Inactivation of adrenaline. Adrenaline is believed to pass continuously into the blood stream from the medullary tissue of the suprarenal gland. Recent work has made it probable that adrenaline is also liberated as the transmitter substance at the adrenergic nerve endings of the sympathetic system [Loewi & Navratil, 1926; Gaddum & Schild, 1934]. After serving its physiological purpose adrenaline is rapidly destroyed; but it is not known how this destruction takes place.

Adrenaline is very readily autoxidizable *in vitro*. The assumption has therefore often been made that adrenaline is also destroyed by autoxidation in the body; but this has been made improbable, since it has been shown that the tissues contain an inhibitor which is very efficient in protecting adrenaline from autoxidation [Wiltshire, 1931; Welch, 1934; Heard & Welch, 1935].

Blaschko *et al.* [1936; 1937] have recently shown that adrenaline is destroyed by an enzyme, adrenaline oxidase, which is present mainly in liver, kidney and intestine. This enzyme is not inhibited by cyanide and oxidizes the adrenaline molecule in the side chain.

In the present investigation adrenaline was found to be rapidly oxidized by cytochrome and by the indophenol oxidase-cytochrome system. The oxidation product adrenochrome, although active as an oxidation carrier, was comparatively inactive as a vasoconstrictor. The indophenol oxidase-cytochrome system is known to be present in practically all animal tissues. This suggests an analogy with the choline esterase system which inactivates the transmitter substance of the cholinergic nerve endings.

Of the sympathomimetic drugs which have been tested it is significant that those which are known to be most rapidly destroyed *in vivo* were also the most rapidly oxidized by the indophenol oxidase-cytochrome system.

Physiological actions of adrenaline. It has been shown qualitatively that under certain conditions adrenaline is rapidly converted into adrenochrome which can act as an oxidation carrier for the coenzyme I dehydrogenase systems of lactic and malic acids. The question arises whether this action of adrenochrome can be effective at concentrations which are of physiological significance.

Stewart & Rogoff [1922] estimated the normal concentration of adrenaline in the blood of the carotid artery as 10^{-9}. In the suprarenal vein they found normal concentrations of about 2×10^{-6} which rose to 10^{-5} after an injection of strychnine. The human suprarenal medullas contain about 9 mg. of adrenaline which in muscular fatigue can be discharged within a few hours. If it is taken that 1 mg. can be discharged in the course of a short spell of muscular effort this represents a concentration of 2×10^{-7} in 5 litres of blood.

In the experiments described on the malic dehydrogenase system adrenaline exerted a carrier action that was measurable *in vitro* at concentrations down to 6×10^{-7}. It is hardly to be expected that the conditions of these experiments were physiologically optimal. The general experience with oxygen carriers such as cytochrome and lactoflavin is that far higher concentrations are required to obtain an oxygen uptake measurable *in vitro* than are necessary in the living cell. It can therefore be concluded that the carrier action of adrenochrome may come within the range of possible physiological concentrations. In reconstructing the complete system *in vitro*, cyanide was necessary for the removal of oxaloacetic acid; but the essential stages of the oxidation could also be carried out individually in the absence of cyanide. The enzymes and substances described are known to be present in mammalian muscle, and there is no known reason why the reactions described should not also occur *in vivo*.

Clearly the carrier action described can have nothing to do with the vasoconstrictor action of adrenaline which is shared by many other phenylethylamine derivatives; but adrenaline has a number of other actions affecting metabolic processes in which the coenzyme and dehydrogenase systems are intimately concerned. The question whether any part of the physiological action of adrenaline can be attributed to the carrier action of adrenochrome remains for further investigation to decide.

SUMMARY

Adrenaline induces a vigorous oxygen uptake when added in low concentrations to the reconstructed lactic and malic dehydrogenase systems of heart muscle. This effect has been analysed and found to be due to the formation of a red-coloured oxidation product, adrenochrome, which can act as a respiration carrier.

Adrenochrome has been isolated in the crystalline state and its properties and constitution have been investigated. A bromo-derivative and an oxime of adrenochrome are described.

The oxidation of adrenaline to adrenochrome is catalysed (1) by a cyanide-insensitive system present in heart and skeletal muscle, and (2) by the cytochrome-indophenol oxidase system present in all tissues.

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Note added 2 April 1937. Since going to press our attention has been drawn to some work of Weinstein & Manning [1935] who obtained a crystalline red product by oxidising adrenaline with silver oxide. This substance they believed to be the *ortho*-quinone of adrenaline; but they give no analytical data, and in view of the work of Ball & Chen [1933] it appears likely that their product is the same as the compound we have described as adrenochrome. It may also be mentioned that Friedheim [1933] found the respiration of rabbits' red blood corpuscles to be increased by 50 % by addition of a red compound obtained by oxidising adrenaline.