THE EARLY STAGES OF THE OXIDATION OF ADRENALINE IN DILUTE SOLUTION

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Summary

The auto-oxidation of adrenaline in concentrations of M/500 to M/10,000 and at pH 6-8 starts after a time lag that is dependent on the conditions but independent of the adrenaline concentration. This is followed by a short period of rapid acceleration of the oxidation rate and then by a period of steady oxidation of constant rate for several hours, proportional to the adrenaline concentration. The solutions are red, later orange to brown, melanin is formed, and the oxygen uptakes, six to seven atoms for one molecule of adrenaline, indicate destructive oxidation beyond opening of the ring structure.

It is suggested that during the time lag the oxidation of adrenaline is started by traces of metallic ions (Fe and Cu) present, adrenochrome being formed in the process and further catalysing the reaction. The acceleration of the oxidation rate and the onset of destructive oxidation are due to the formation of more readily oxidizable compounds of indoxyl structure, the reaction probably involving a free radical mechanism. The later, constant oxidation proceeds over the quinonoid redox systems of adrenochrome and oxo-adrenochrome that are established during the period of acceleration. At this stage the reaction mixture consists of unreacted adrenaline, the quinonoid systems, and of further oxidation products, which act as hydrogen donors towards the quinones. The regulation of the oxidation rate is ascribed to the effect of the hydrogen transfers on the redox potential of the quinonoid systems and possibly to the inhibiting influence of later oxidation products. Finally the ring structures are consumed and, apart from condensation products like melanin, the reaction mixture consists of late oxidation products which without the action of redox systems, undergo further atmospheric oxidation. Tentative formulations of the earlier steps of adrenaline oxidation are proposed.

The influence of catalysts, heavy metal ions (Fe and Cu, also haematin and methaemoglobin), and adrenochrome is confined to the initial stages of the reaction sequence; they shorten the time lag and cause a transient period of more rapid oxygen uptake, the duration of which depends on their concentration. In catalytic amounts they do not appreciably affect the rate of the final oxidation.

The auto-oxidation of adrenochrome begins after a time lag and shows a period of more rapid oxidation before the final steady oxidation rate is established.

Oxidizing red adrenaline solutions or adrenochrome solutions form strongly fluorescent colourless to yellow solutions on reduction and on addition of free alkali with or without the admission of air. It is suggested that, depending on the conditions, leuco-adrenochrome or *N*-methyl-5 : 6-dihydroxy-indoxyl, but usually mixtures of both compounds are formed. If at lower pH (7-8.5) adrenaline is allowed to oxidize in the presence of reducing agents (sulphite,

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amino acids, etc.) almost colourless fluorescent solutions are formed; adrenochrome is quickly changed by serum into a green fluorescent solution. The question whether the reaction mixture contains the red quinones or the fluorescent leuco compounds depends on the proportion of primary oxidation to dehydrogenation.

Most polyphenolases oxidize adrenaline beyond the adrenochrome stage. Several preparations from the solanaceous plant *Duboisia myoporoides* were found to cause an uptake of two atoms of oxygen only.

The presence of a peroxidative step in the reaction sequence of adrenaline oxidation and the effect of catalase are mentioned.

In the presence of free alkali, adrenaline rapidly consumes six, adrenochrome four atoms of oxygen per molecule, clear yellow, strongly fluorescent solutions being formed. This period of rapid oxygen uptake is followed by a prolonged period of slow uptake, two to three more atoms of oxygen being consumed. During this period the solutions are clear yellow but without any fluorescence. Neither red quinones nor melanin are formed under these conditions.

Age, colour, fluorescence, or oxygen uptakes of an oxidizing adrenaline solution can give no reliable indication as to its composition. If the physiological action of an adrenaline oxidation product is to be investigated, synthetic intermediates will have to be chosen. Tentative methods for the preparation of comparatively pure solutions of the fluorescent compounds are proposed.

I. Introduction

In recent years several publications have described physiological effects of somewhat indefinite oxidation products of adrenaline, or merely of "aged solutions," "oxidized adrenaline," or "coloured adrenaline" (cf. review by Bacq 1949). The observations often appear contradictory and are not always reproducible; their interpretation presents difficulties since the composition of the solutions has not been ascertained.

The first steps of adrenaline oxidation are usually presented as proceeding from adrenaline to adrenaline-quinone, leuco-adrenochrome, and adrenochrome (Fig. 22, I-IV†). Doubts concerning this sequence have been expressed by West (1947), Bacq (1949), and others, and will be discussed later. A formulation of the mechanism of the oxidation beyond adrenochrome has not yet been proposed. The oxidation of adrenaline has been investigated with the purpose of establishing to what degree colour, fluorescence, oxygen uptakes, or age of an oxidizing solution give an indication of its composition. The mechanism of adrenaline oxidation at physiological pH is presented as proceeding first to *o*-quinones and the corresponding diphenols. It is suggested that the destructive oxidation mechanism starts through disproportionation of free radicals and proceeds further by hydrogen transfer from later oxidation products to the quinones.

† I, Adrenaline; II, adrenaline-quinone; III, leuco-adrenochrome; IV, adrenochrome; IV*a*, quinone-immonium structure of adrenochrome in acid solution; IV*, semiquinone of the adrenochrome system; IV**, hypothetical di-radical; V, *N*-methyl-5 : 6-dihydroxy-indoxyl; VI, oxo-adrenochrome; VII, *N*-methyl-5 : 6-dihydroxy-indole.

II. Materials and Methods

Crystalline adrenaline was supplied by H. Francis and Co., Melbourne. The preparations showed the correct melting point (211-212°C.) and the experimental results were not modified by further purification.

Adrenochrome was prepared by the method of Randall (1946).

Polyphenolase from the fresh leaves of the solanaceous plant *Duboisia myoporoides* was prepared as described by Trautner and Roberts (1950).

Catalase was prepared from rat liver homogenized with 10 volumes of M/100 phosphate buffer, pH 7.0, and centrifuged. One ml. of a 1 : 50 dilution of the supernatant was used. In some experiments crystalline beef liver catalase prepared by the method of Sumner and Dounce (1939) was reacted in parallel experiments.

Oxygen uptakes were measured in Warburg manometers under the conditions described. Final fluid volume was 5 ml. in all experiments. To allow of better comparison of the results the graphs are, as far as practicable, scaled for oxygen uptakes corresponding to 1 ml. M/100 solution of the substrate to be oxidized (adrenaline, adrenochrome, etc.).

Table 1

VISIBLE CHANGES ON SLOW ATMOSPHERIC OXIDATION OF M/500 ADRENALINE IN M/100 BUFFER OR SALT SOLUTION IN TEST TUBES WITH OCCASIONAL AGITATION ON STANDING AT ROOM TEMPERATURE (23°C.)

Time	H_3PO_4	NaH_2PO_4	Buffer pH 8.0	Na_2HPO_4	$NaHCO_3$	Na_2CO_3	NaOH
On mixing	None	None	None	None	Pink	Pink	Pink
5 min.	None	None	None	Pink	Pink	Yellow	Yellow
1 hr.	None	None	Pink	Pink	Pink	Yellow	Yellow
3 hr.	None	None	*Orange*	*Orange*	Yellow	Yellow	Yellow
5 hr.	None	None	*Red*	*Red-brown*	Yellow	Yellow	Yellow
20 hr.	None	None	*Dark brown*	*Brown*	Yellow	Yellow	Yellow

Volatile amines evolved during oxidation of adrenaline or adrenochrome were determined by the diffusion method of Conway (1947), using boric acid mixture in the centre well.

Colour density was measured with a Lumetron colorimeter (Photovolt, New York), using a green filter for adrenochrome according to the method of Evans and Raper (1937).

Fluorescence was measured with a Klett fluorimeter against eosin solutions as standard, as suggested by West (1947).

All reagents used were of reagent quality. Fresh solutions were prepared for each experiment and the experiments were put on as soon as the flasks were ready. Since the oxidation of adrenaline is strongly catalysed by heavy metal ions, the vessels were washed with glass-distilled water and alcohol and finally steamed. Care was taken to avoid contact of the substances or solutions with metal surfaces, spatulas, etc. Under these conditions reproducible results were obtained.

III. Experimental Results

(*a*) *Oxidation of Adrenaline*

(i) *Non-enzymic oxidation*

Table 1 presents the visible changes observed on slow atmospheric oxidation of M/100 solutions of adrenaline under different conditions at room temperature and with occasional agitation. Higher temperature, stronger aeration, and the presence of catalysts accelerate the development of the changes.

Figure 1 shows the oxygen uptakes of M/2500 adrenaline at different pH levels.

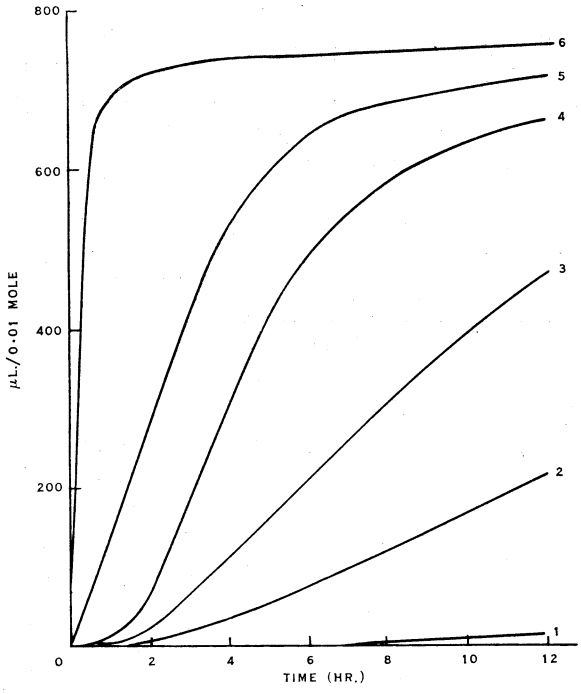

Fig. 1.—Auto-oxidation of adrenaline at various pH. Final adrenaline concentration M/2500.
Curve 1: 0.1N phosphoric acid at 30°C.
Curve 2: M/15 phosphate buffer, pH 7.0; 23°C.
Curve 3: M/15 phosphate buffer, pH 7.0; 30°C.
Curve 4: M/15 phosphate buffer, pH 8.0; 30°C.
Curve 5: M/15 barbiturate buffer, pH 9.0; 30°C.
Curve 6: 1N caustic soda; 30°C.

Oxidation at alkaline pH.—(1) On oxidation of dilute solutions at pH values over 11 the red colour of adrenochrome is not observed. The solutions turn yellow on admixture of the alkali and show a transient fluorescence, which will be discussed separately. With higher concentrations of adrenaline, turbid brown

solutions are finally obtained, but at the dilutions used the solutions remain clear and neither turbidity nor precipitates are formed. The final pale canary-coloured solutions appear to remain unchanged for days. They do not seem to have any physiological action (Shaw 1941).

The oxygen uptakes show an initial high oxidation rate leading rapidly to the uptake of six atoms of oxygen for one molecule of adrenaline; this period of rapid oxidation is followed by a prolonged period of slow oxidation. No changes in the oxidation rate could be observed at oxidation levels corresponding to the formation of leuco-adrenochrome (two atoms of oxygen); *the alkaline oxidation of adrenaline up to an uptake of six atoms of oxygen proceeds as a steady, continuous function and not in distinct successive steps.* The period of slow oxidation may extend over as much as 24 hours. Owing to the prolonged time of this further reaction, exact measurements of the oxygen uptakes are not possible, but at least two and possibly over three further atoms of oxygen are slowly consumed. No visible changes can be observed during this later period; the solutions remain clear canary yellow.

(2) The reaction mixtures were submitted only to qualitative tests with the purpose of establishing whether the final yellow solutions obtained by different procedures can be regarded as identical. The early fluorescent solutions give positive tests for phenols (ferric chloride, coupling with diazo compounds) and for indole or indoxyl grouping (Ninberg's ninhydrin test, Ehrlich's dimethyl-aminobenzaldehyde test, the formalin-sulphuric acid test). With the uptake of about six atoms of oxygen for one molecule of adrenaline, the last traces of fluorescence disappear and these tests become negative. Later, ferric chloride gives temporarily a wine-red colour similar to the colour in Gerhard's test for aceto-acetic acid; at the same time a weak Rothera test with nitroprusside is shown, both suggesting the temporary presence of β-keto acids (β-keto-adipic acid has been isolated as a product of bacterial oxidation of aromatic compounds by Kilby 1949). Still later, these tests also become negative and, apart from the change of the yellow colour to a faint pink on acidification, the only simple reaction found was the formation of a slight turbidity with calcium chloride (catechol in alkaline solution is oxidized by permanganate to oxalic acid amongst other products). Part of the nitrogen is finally present in the form of volatile amines; diffusion in Conway units showed up to 50 per cent. adrenaline nitrogen diffusing into the centre well within 24 hours at room temperature on alkaline oxidation.

Oxidation in phosphate buffer at physiological pH.—The solutions obtained are unlike those formed on alkaline oxidation, since under all conditions of oxidation a strong red colour is formed. At intermediate pH, between 10 and 11, this colour is short-lived and soon changes to orange or yellow solutions identical with those of alkaline oxidation. At more neutral pH the colour persists, at first clear and increasing in intensity to a brilliant dark red; after 1-1½ hours, however, it loses its brilliancy and becomes orange-brown to dull brown. Finally, brown solutions or suspensions are formed, which even on prolonged

standing do not appear to undergo any further changes. In Table I the observations indicative of the presence of coloured compounds, orange, red, or brown, are in italics; they are confined to pH's between 7 and 8.

(1) Auto-oxidation.—Figure 1, curves 3 to 5, presents the oxygen uptakes of M/2500 adrenaline at pH 7, 8, and 9 at 30°C. The reaction is auto-catalytic and starts after a time lag that amounts to 1-3 hours or more, depending on the conditions. The duration of this lag appears to be, within wide limits, *independent of the concentration of adrenaline* (Fig. 2). Almost abruptly the oxidation rate begins to increase. A constant rate is established after the uptake of about one-half atom of oxygen for one molecule of adrenaline; its magnitude *depends on the concentration of adrenaline* (Fig. 3). The constant rate is maintained for several hours. The oxygen uptakes exceed the adrenochrome level—two atoms for one molecule of adrenaline—and reach four to five atoms before the curves begin to flatten out. The curves do not show the distinct six-atom level observed on alkaline oxidation; rather they appear to exceed it in a smooth, continuous line. The reaction gradually slows down and the total oxygen uptake is between seven and eight atoms, slightly less than that of alkaline oxidation. At higher pH, the curves are S-shaped, showing three distinct sections: the time lag, a period of rapid oxidation, and then the final period of slow oxidation (Fig. 1, curve 4).

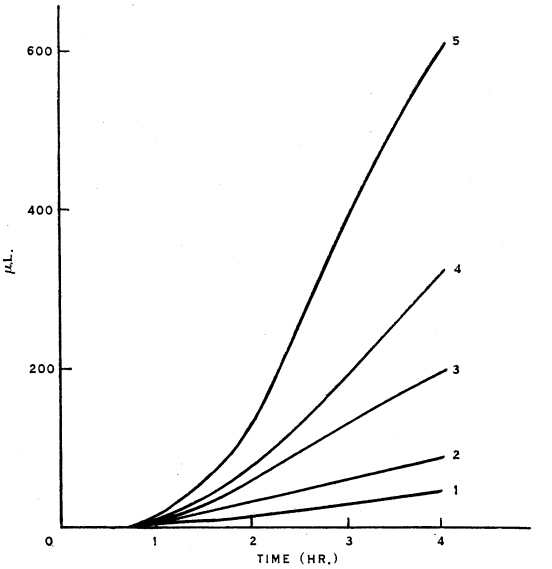

Fig. 2.—Auto-oxidation of adrenaline at various concentrations. M/15 phosphate buffer, pH 8.0; 30°C.
Curve 1: Adrenaline final concentration M/4000.
Curve 2: Adrenaline final concentration M/2000.
Curve 3: Adrenaline final concentration M/1000.
Curve 4: Adrenaline final concentration M/500.
Curve 5: Adrenaline final concentration M/250.

(2) Metal catalysis.—The early phases of the oxidation of adrenaline at physiological pH are strongly catalysed by heavy metal ions, particularly ferric and cupric ion. Figure 4 shows the oxygen uptakes recorded under otherwise identical conditions in the presence of different concentrations of metallic ion. The effect of low concentrations of the catalyst consists solely in shortening the time lag; the rate of constant oxidation finally reached remains unaltered. With increasing concentrations of catalyst there appears, *between the time lag and the later period of constant oxidation,* a period of more rapid oxidation, the duration of which increases with the concentration of the metallic ion. The final rate of constant oxidation is almost the same whether metallic ion has been added to the solution or not. Identical series of curves have been obtained by Falk (1949) and Slater (1949) by using various concentrations of haematin or methaemoglobin as catalysts; the latter author noted that the final oxidation rate is not noticeably affected by the amount of catalyst added. It appears also to be independent of the nature of the catalyst as well as of the amounts of oxygen consumed during the intermediate period of rapid oxidation.

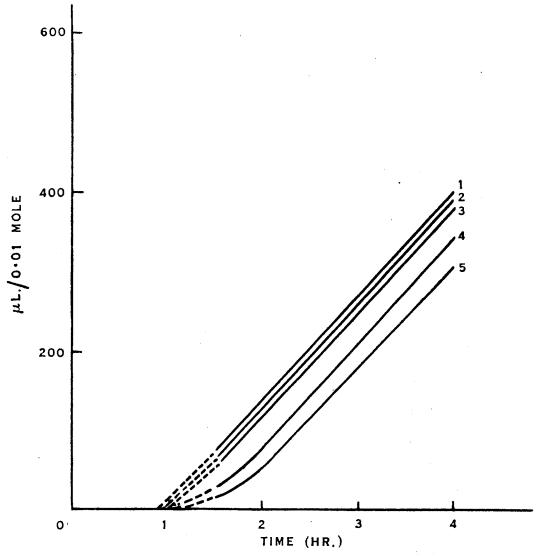

Fig. 3.—Molar oxidation rate. Same experiment as shown in Figure 2; oxygen uptakes plotted for 0.01M adrenaline.

(3) Adrenochrome catalysis.—The addition of adrenochrome, though in 10-20 times the concentration of the metallic ions, causes an identical shortening of the time lag and, possibly, a slight initial acceleration of the oxidation rate (Fig. 5). The final rate of oxidation is not essentially altered. The effect of the catalyst appears to be confined to the first stages of the oxidation. At a proportion of adrenochrome : adrenaline between 1 : 5 and 1 : 10, the constant rate of oxidation is established almost directly after a scarcely noticeable time lag or period of rapid oxidation (Fig. 5, curve 3).

(4) Composition of the solutions.—Apart from the differences in the onset of oxidation and the changes in oxidation rates, the composition of the solutions appears to be the same whether the oxidation has been conducted in the absence or presence of catalysts, and again whether the catalyst was iron, copper, or adrenochrome. Only a few observations are pertinent to the investigation in hand.

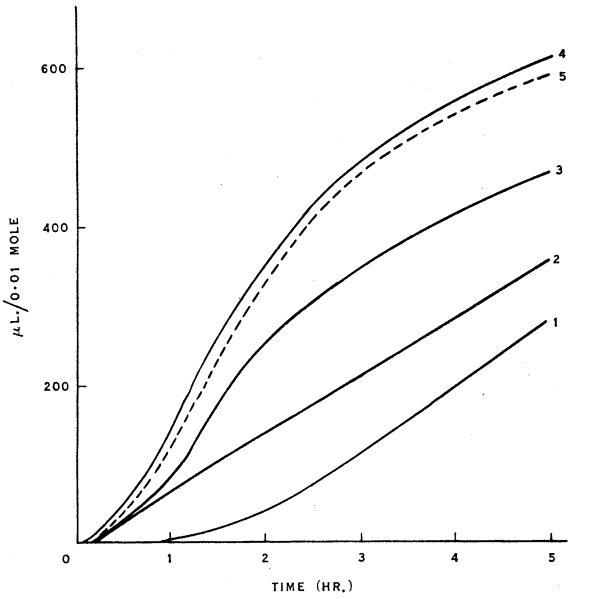

Fig. 4.—Adrenaline oxidation. Metal catalysis. Final adrenaline concentration M/2500; M/15 phosphate buffer, pH 8.0; 37°C.

Curve 1: Adrenaline alone.

Curve 2: Adrenaline with $CuSO_4$ in final concentration of M/50,000.

Curve 3: Adrenaline with $CuSO_4$ in final concentration of M/25,000.

Curve 4: Adrenaline with $CuSO_4$ in final concentration of M/5000.

Curve 5: Adrenaline with $FeCl_3$ in final concentration of M/5000.

The first visible sign of beginning oxidation consists in the formation of a pink tint; the colour increases quickly to a clear red. These red solutions give quinone reactions and adrenochrome can be separated from the reaction mixture (Green and Richter 1937; Richter and Blaschko 1937). Colorimetric estimation of the adrenochrome present, practicable only so long as the solutions are clear, shows concentrations far below those of an adrenochrome solution equimolecular to the amount of adrenaline initially present. The method — used by Fischer and Lecomte (1949) for the determination of adrenochrome secreted in urine—is not considered very reliable since adrenochrome solutions alone

show slowly increasing colour intensity on standing. The values are probably still too high and indicate only that adrenochrome is not quantitatively formed at an early stage of the oxidation and that brown "melanin" is already formed before the total amount of adrenaline present is oxidized to the adrenochrome stage.

In these turbid brown solutions further oxidation proceeds to an oxygen uptake of seven to eight atoms of oxygen for one molecule of adrenaline, i.e. to the destructive oxidation beyond ring opening of at least a major fraction of the total adrenaline present. The pH of an unbuffered, oxidizing adrenaline solution begins to drop after a few hours and reaches values between pH 4 and 5, indicating the formation of acidic oxidation products (Fig. 6*A*).

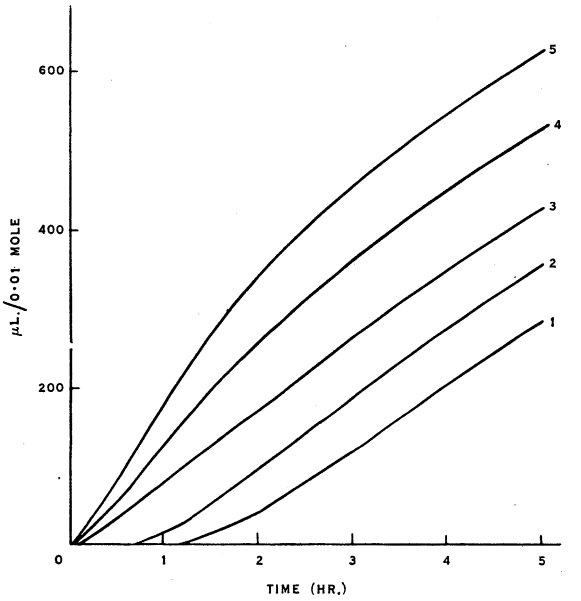

Fig. 5.—Adrenaline oxidation. Adrenochrome catalysis. Final adrenaline concentration M/2500; M/15 phosphate buffer, pH 7.7; 37°C.
Curve 1: Adrenaline alone.
Curve 2: Adrenaline with 10 per cent. adrenochrome.
Curve 3: Adrenaline with 20 per cent. adrenochrome.
Curve 4: Adrenaline with 40 per cent. adrenochrome.
Curve 5: Adrenaline with 80 per cent. adrenochrome.

Hydrogen peroxide is formed during the oxidation of adrenaline (Schales 1938). Hartung (1946) and Falk (1949) suggested that it is formed in the oxidation of leuco-adrenochrome to adrenochrome; no evidence for this location was found in the present work. Figure 7 shows the effect of the addition of catalase to the oxidizing solutions. The amount of oxygen evolved increases as the reaction proceeds, suggesting that *the peroxide-forming step persists*

throughout the whole period of oxidation and is not limited to a distinct early stage. It is doubtful whether hydrogen peroxide plays an essential role in the reaction sequence of the auto-oxidation of adrenaline since the addition of hydrogen peroxide scarcely affects it and that of catalase merely slightly delays it (Fig. 8). The oxidation of adrenaline in the presence of catalase and catalase-containing body fluids is under separate investigation.

If the early clear red solutions are rendered alkaline, the oxidation rate increases instantaneously and the oxygen uptakes follow the pattern of alkaline oxidation leading to clear yellow final solutions. If, however, the later turbid brown solutions are rendered alkaline, the rate and amount of oxygen taken up are less and the solutions remain dirty brown. Once formed these melanins appear to be fairly resistant to further oxidation.

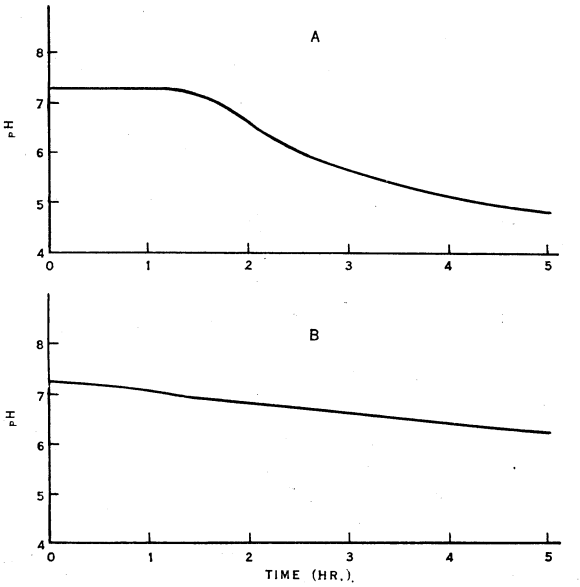

Fig. 6.—*A.* Changes in pH during adrenaline auto-oxidation. M/100 adrenaline was adjusted to pH 7.2 with a few drops of 0.001N NaOH; 20°C. No buffer was added and oxygen was bubbled through the solution throughout the experiment.

B. Changes in pH during adrenochrome auto-oxidation; same conditions.

Oxidation at acidic pH.—At acidic pH, below 6, the auto-oxidation of adrenaline is very slow and no visible changes or noticeable oxygen uptakes may be observed for a day or more. If a suitable catalyst or oxidizing agent is added, oxidation and colour formation occur. The authors agree, however, with Bacq (1949) that not every red colour observed should be accepted as being due to adrenochrome. For example, the initial red formed at pH 3 by iodate, cited by Bacq, Fischer, and Lecomte (1948) as being inhibited by di-

mercaptopropanol (BAL), soon changes into a stable, deep violet pigment, iodoadrenochrome (Richter and Blaschko 1937). The red colour formed at the same pH by persulphate is due to oxo-adrenochrome, synthesized by Cohen (1945). The conditions of the acidic oxidation of adrenaline were not investigated in detail.

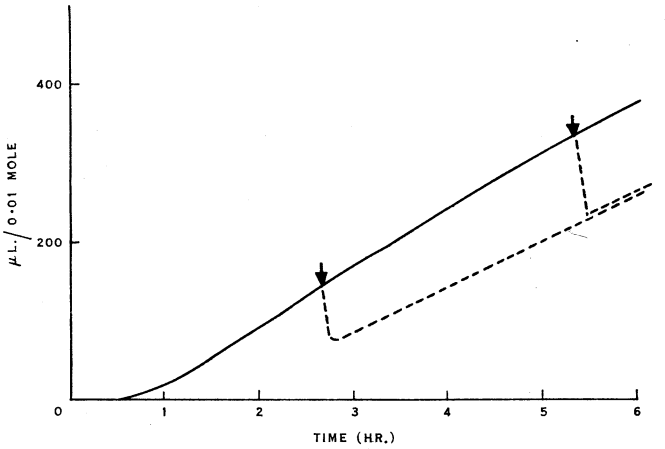

Fig. 7.—Effect of the addition of catalase to adrenaline oxidation mixtures. Final adrenaline concentration M/2500; M/15 phosphate buffer, pH 8.0; 30°C. Catalase added at arrows, 1 ml. of 1 : 50 diluted liver supernatant.

(ii) *Enzymic Oxidation*

The enzymic oxidation by a polyphenolase at physiological pH is usually quoted as consuming considerably more oxygen than corresponds to the adrenochrome level (cf. James *et al.* 1948). Bacq (1949) says that the auto-oxidation of adrenaline *in vitro* is presumably comparable in its chemical mechanism to its oxidation by catechol oxidase. The present authors obtained, however, acetone powders from the solanaceous plant *Duboisia myoporoides*, which between pH 5.5 and 7.8 and at temperatures between 23° and 30°C. consumed exactly *two atoms of oxygen for one molecule of adrenaline* (Fig. 9, curve 6; Fig. 10, curve 2). The shape of the curves is almost identical with that obtained for the same concentrations of catechol and the final solutions after complete oxidation, 1-1½ hours, are not brown like those obtained by catalytic or auto-oxidation under the same conditions, but clear deep red of a colour intensity equal to, or only slightly less than, that of equimolecular fresh adrenochrome solutions. No difference could be found between them and adrenochrome solutions when in many experiments the enzymic preparation, filtered from the insoluble enzyme, was substituted for adrenochrome solutions of equal concentration.

Not all enzyme preparations, however, showed this theoretical oxidation to adrenochrome; many lead to solutions identical with those obtained on auto-oxidation *except for a higher initial rate of oxygen uptake.* Figure 9 presents the oxygen uptakes of such an "inferior" enzyme at various pH; it is seen that

at pH 5.5 (where the time lag of auto-catalysis may extend to several hours) the oxygen uptake is almost theoretical (Holtz and Kroneberg 1950) but at higher pH a steady rate of further oxidation is maintained *after* the initial period of rapid oxidation. It would appear that enzymic and non-enzymic oxidation are superimposed. No time lag is noticeable in the enzymic oxidation.

Attempts to control the action of an "inferior" enzyme were without success. It was not possible to limit its action to the two-atom level by the addition of catalase, or to arrest the mechanism of further oxidation by the later addition of a good enzyme to the reaction mixture. It would appear that once the mechanism of destructive oxidation has started it follows its course. It was not possible to find any explanation why some enzyme preparations were superior to others.

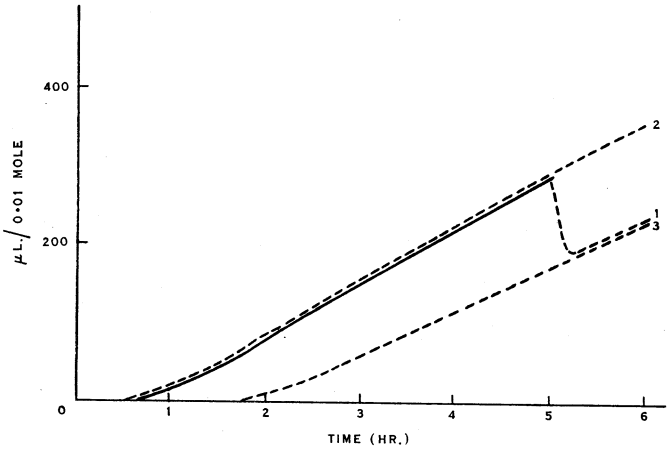

Fig. 8.—Effect of the presence of H_2O_2 or catalase on the course of adrenaline oxidation. Final adrenaline concentration M/2500; M/15 phosphate buffer, pH 8.0; 30°C.
Curve 1: Adrenaline alone; catalase added at end of solid line.
Curve 2: Adrenaline plus 1 ml. M/100 H_2O_2.
Curve 3: Adrenaline plus catalase, 1 ml. 1 : 50 liver supernatant.

(*b*) *Oxidation of Adrenochrome*

(i) *Non-Enzymic Oxidation*

Table 2 shows the visible changes observed if dilute solutions of adrenochrome (M/500) are allowed to oxidize at room temperature under conditions identical with those shown for adrenaline.

Oxidation at alkaline pH.—Instantaneously on admixture of alkali, adrenochrome loses its red colour; a yellow, strongly fluorescent solution is formed and rapid oxidation sets in. The general shape of the curves of oxygen uptakes is identical with those observed with adrenaline under the same conditions except that two fewer atoms of oxygen are consumed *during the initial period of rapid oxidation* (Fig. 10, curve 1). If adrenaline is first oxidized enzymically to adrenochrome at pH 7.7 and then free alkali added to the reaction mixture, the same oxidation level is reached as if the adrenaline had been directly

oxidized by free alkali (Fig. 10, curves 2 and 3). The rapid initial oxidation is followed by a prolonged, very slow oxygen uptake extending over 24 or more hours, as with adrenaline. The final yellow non-fluorescent solutions were found to be identical, in all tests applied, with those obtained in the alkaline oxidation of adrenaline.

It was investigated whether the formation of the yellow fluorescent compound from adrenochrome on admixture of alkali was due to oxidation. Oxygen was excluded by placing adrenochrome solutions in Thunberg tubes with alkali in the side-arm and evacuating with continuous tapping for as long as 30 minutes before mixing the contents. Under no conditions was it possible to obtain

TABLE 2

VISIBLE CHANGES ON SLOW ATMOSPHERIC OXIDATION OF M/500 ADRENOCHROME IN M/100 BUFFER OR SALT SOLUTION WITH OCCASIONAL AGITATION AT ROOM TEMPERATURE (23°C.)

Time	Dil. HCl	Dil. H_3PO_4	NaH_2PO_4	Buffer pH 8.0	Na_2CO_3	NaOH
On mixing	Slight fading	Slight fading	No change (red)	No change	No change	Yellow fluores.
10 min.	Dirty green	Dirty green	No change (red)	No change	Greenish fluores.	Yellow
2 hr.	Dirty green	Dirty green	No change (red)	No change	Yellow	Yellow
18 hr.	Dirty green and dark ppt.	Dirty green and dark ppt.	Light green-brown and ppt.	No change	Yellow-brown	Yellow
48 hr.	Dirty green and dark ppt.	Dirty green and dark ppt.	Light green-brown and ppt.	No change	Brown	Yellow
64 hr.	Dirty green and dark ppt.	Dirty green and dark ppt.	Light green-brown and ppt.	Pale brown	Brown	Yellow

a red solution of adrenochrome at alkaline pH; *no oxygen appears to be required for the formation of the fluorescence.* To establish whether the change was reversible, the experiments were repeated in Thunberg tubes with two side-arms, the second containing phosphoric acid equivalent to the amount of alkali in the first so as to bring the alkaline mixture back to neutrality without admission of air. *The change into "fluorescent adrenochrome" is irreversible;* the neutralized solutions are yellow to greenish, strongly fluorescent, and moderately stable. On admission of air they take up oxygen slowly, though faster than adrenaline. The oxygen uptakes on alkalization are identical with those of the original adrenochrome solution, as could be expected. The maximum intensity of fluorescence is of the same order as that observed on alkalization in the presence of air.

Oxidation in phosphate buffer at physiological pH.—The auto-oxidation of adrenochrome proceeds similarly to that of adrenaline but the time lag is shorter and *a temporary early period of rapid oxidation is observed* (Fig. 11).

This latter appears to be characteristic of adrenochrome oxidation. The constant oxidation rate finally reached is slightly lower than that of equimolecular adrenaline solutions. The presence of small amounts of adrenaline exercises a catalytic effect similar to that of small amounts of adrenochrome on adrenaline oxidation: the time lag is shortened; the rate of initial oxidation is possibly slightly increased; but the rate of later oxidation is not essentially altered. In

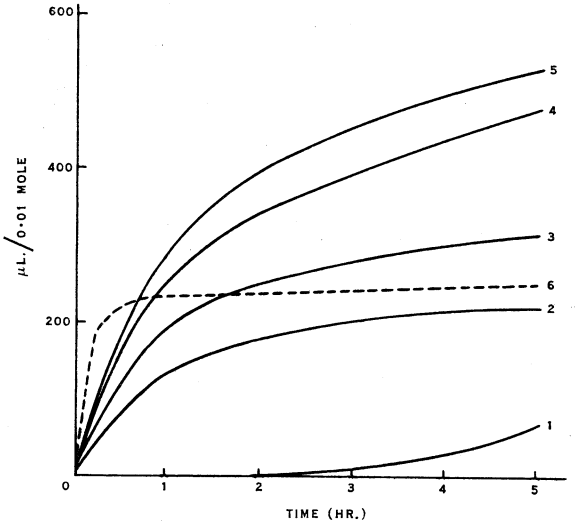

Fig. 9.—Enzymic oxidation of adrenaline. Final adrenaline concentration M/2500; M/15 phosphate buffer; 23°C. Curves 1-5 show the oxygen uptakes with *Duboisia* polyphenolase, 30 mg., at various pH; curve 6 the uptake with a "purer" enzyme (see text).
Curve 1: Adrenaline alone, pH 7.0.
Curve 2: Adrenaline plus enzyme, pH 5.5.
Curve 3: Adrenaline plus enzyme, pH 6.0.
Curve 4: Adrenaline plus enzyme, pH 7.0.
Curve 5: Adrenaline plus enzyme, pH 8.0.
Curve 6: Adrenaline plus "purer" enzyme, pH 7.0.

proportions of adrenochrome to adrenaline between 1 : 5 and 5 : 1 the same initial catalytic effect is observed but the rate of constant oxidation finally reached is considerably less than the sum of those of the components if singly reacted. The mixtures establish an intermediate oxidation rate (Fig. 12). It would appear that the catalytic effect is confined to the early phases of the reaction.

The pH of an oxidizing, unbuffered adrenochrome solution begins to drop earlier than observed with adrenaline (Fig. 6*B*).

Oxidation at acidic pH.—Between pH 3 and 4 adrenochrome is within ½-1 hour converted into a *green compound*, the change soon becoming irreversible; neutralization no longer restores any red colour, but merely a faint pink tint; on alkalization a slight fluorescence is observed, but the oxygen uptakes

are slower and less than those on direct alkalization of adrenochrome. On standing at acidic pH these green solutions deposit within 24 hours a black, unreactive precipitate. Whether this "black melanin" is identical with the "brown melanins" formed at physiological pH was not investigated, nor could it be established whether its formation is accompanied by oxidation. During the first few hours at least *no oxygen is consumed in the formation of "green adrenochrome"* (Fig. 11, curve 1). The instability of red adrenochrome in acid solution makes it still more doubtful whether any red colours observed on acid oxidation of adrenaline are due to adrenochrome. The oxidation of adrenaline to "green adrenochrome" with later formation of black melanin can be effected by acid permanganate.

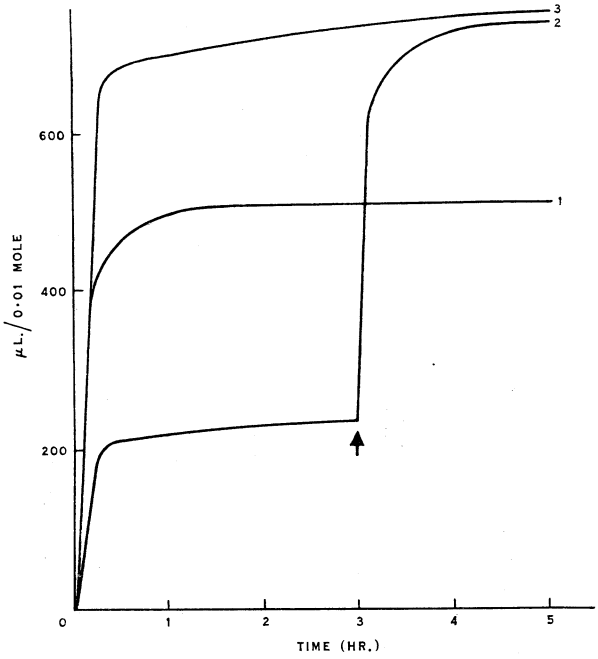

Fig. 10.—Adrenochrome: alkaline oxidation. Final adrenochrome and adrenaline concentrations M/2500; 25°C.

Curve 1: Adrenochrome with 1N NaOH.

Curve 2: Adrenaline first oxidized at pH 7.0 with 30 mg. of a "purer" enzyme, 1 ml. 1N NaOH added at arrow.

Curve 3: Adrenaline with 1N NaOH.

(ii) *Enzymic Oxidation*

The addition of a polyphenolase does not accelerate the auto-oxidation of pure adrenochrome, and no convincing evidence could be obtained that it accelerates that of aged adrenochrome or of the acidic green or the alkaline yellow solutions if they are brought back to physiological pH. It would appear that the enzyme used reacts rapidly with the catechol grouping of the benzene

ring, but only sluggishly or not at all with those of the nitrogen-substituted adrenochrome system and that it has no effect on the mechanism of the further oxidation of these compounds. If traces of adrenaline are present, an initial rapid oxygen uptake is observed, but then the curves follow their usual course.

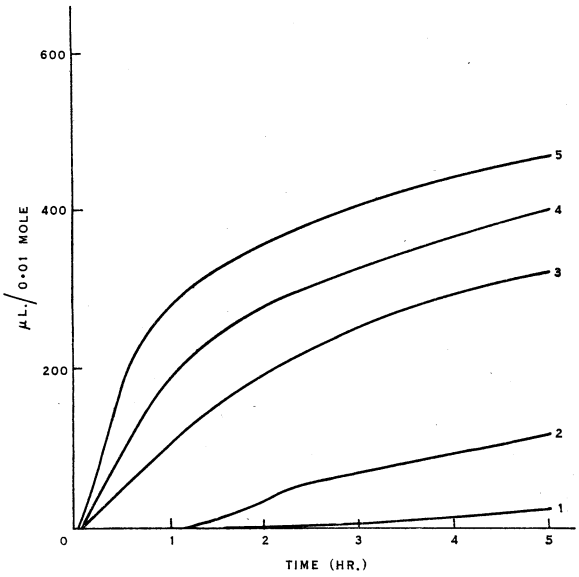

Fig. 11.—Adrenochrome oxidation at various pH. Final adrenochrome concentration M/2500; 37°C.
Curve 1: Adrenochrome in dilute phosphoric acid, pH 4.0.
Curve 2: Adrenochrome in M/15 phosphate buffer, pH 5.0.
Curve 3: Adrenochrome in M/15 phosphate buffer, pH. 6.0.
Curve 4: Adrenochrome in M/15 phosphate buffer, pH 7.0.
Curve 5: Adrenochrome in M/15 phosphate buffer, pH 8.0.

(iii) *Comment on Some Observations on Adrenaline Oxidation*

To shorten the final discussion, three points are separated that are of limited pertinence to the present investigation, namely the enzymic oxidation of adrenaline, the formation of green and fluorescent adrenochrome, and the formation of melanin.

Enzymic oxidation.—The mechanism responsible for the fact that some enzyme preparations stop the oxidation of adrenaline at the two-oxygen stage, with almost quantitative formation of adrenochrome and without any formation of melanin, is outside the scope of this investigation. It is, therefore, only intended to mention the possibilities considered.

(1) Inhibition of metallic catalysts.—Proteins, serum, and other body fluids inhibit the auto-oxidation of adrenaline (Bonhomme 1936). Slater (1949) noticed that small amounts of methaemoglobin inhibit while greater amounts

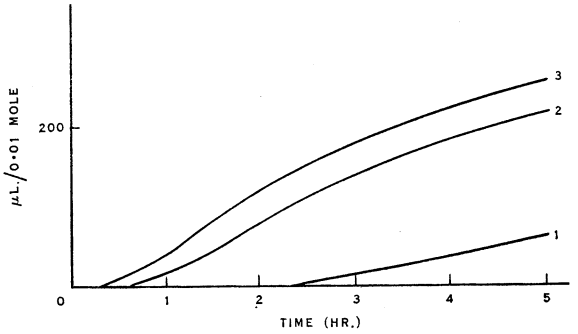

Fig. 12.—Oxidation of an equimolecular mixture of adrenaline and adrenochrome. Final adrenaline and adrenochrome concentrations M/2500; M/15 phosphate buffer, pH 7.0; 25°C.
Curve 1: Adrenaline alone.
Curve 2: Adrenochrome alone.
Curve 3: Adrenaline plus adrenochrome.

catalyse it. It is possible that some component of the acetone powder used combines with and inactivates such traces of metallic ions as are present and necessary to start auto-oxidation. This factor, however, cannot be the only one,

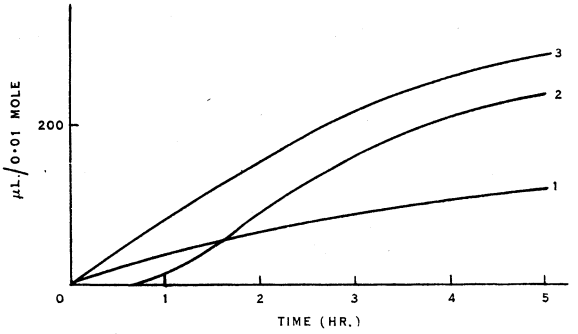

Fig. 13.—Oxidation of an equimolecular mixture of adrenochrome and fluorescent compound. The fluorescent compound was prepared by sodium metabisulphate reduction of adrenochrome, the excess of bisulphite being destroyed by iodine. Final concentration of adrenochrome and fluorescent compound M/2500; M/15 phosphate buffer, pH 7.0; 25°C.
Curve 1: Fluorescent compound alone.
Curve 2: Adrenochrome alone.
Curve 3: Adrenochrome plus fluorescent compound.

since in the action of a good enzyme not only the destructive oxidation caused by metal catalysis but also that caused by adrenochrome catalysis is inhibited.

(2) Lack of hydrogen peroxide.—Nelson and Dawson (1944) in a review on tyrosinase, reach the conclusion that no hydrogen peroxide is formed in the specific action of polyphenolases on phenols and catechols. Since hydrogen peroxide is formed during the auto-oxidation of adrenaline, it may be considered that its presence is essential to start or maintain the mechanism of further and finally destructive oxidation. The suggestion is invalidated by the observation that the addition of hydrogen peroxide does not influence the course of adrenaline auto-oxidation and that of catalase merely delays it (Fig. 8, curves 2 and 3).

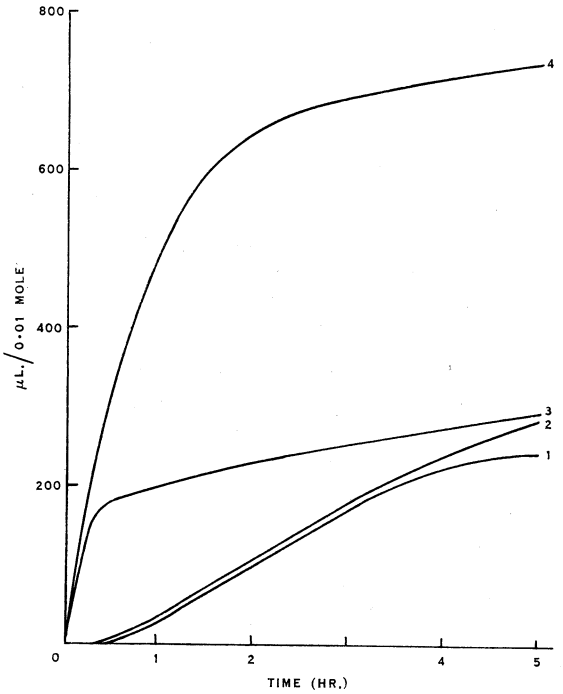

Fig. 14.—Adrenaline oxidation in bicarbonate and phosphate buffers, alone and in the presence of equimolecular amounts of $CuSO_4$. Final adrenaline and copper sulphate concentrations M/2500; pH 7.3; 37°C.
Curve 1: Adrenaline in 0.016N bicarbonate in an atmosphere of 95 per cent. oxygen and 5 per cent. CO_2.
Curve 2: Adrenaline in M/15 phosphate buffer.
Curve 3: Adrenaline plus copper sulphate in bicarbonate as in (1).
Curve 4: Adrenaline plus copper sulphate in phosphate as in (2).

(3) A different course of the reaction.—The early steps of adrenaline (I) oxidation are presented as proceeding from adrenaline to the unstable adrenalinequinone (II), which quickly rearranges itself to leuco-adrenochrome (III),

this in turn reacting with further oxygen to form adrenochrome (IV). The redox potential of the leuco-adrenochrome/adrenochrome system is below that of the adrenaline/adrenaline-quinone system (Wiesner 1942; Falk 1949) (cf. Fig. 22). If during a very rapid enzymic oxidation *adrenaline-quinone is formed at a rate exceeding that of its rearrangement,* it would oxidize leuco-adrenochrome according to the equation in Figure 23 II from right to left. If this is the case, at no time would there be a noticeable concentration of leuco-adrenochrome in the reaction mixture. The suggestion that the presence of leuco-adrenochrome *and* adrenochrome is necessary for the onset of destructive oxidation is supported by the observation that, in adrenochrome catalysis, a time lag is observed (Figs. 12 and 13), indicating that a certain concentration of metabolites is necessary to establish the optimal rate of oxidation. Adrenochrome catalysis would thus seem to be lacking in the enzymic oxidation but to be an essential factor in the destructive oxidation of adrenaline*.

The structures of "green" and of "fluorescent" adrenochrome.—Marquardt (1947) formulated adrenochrome as a zwitterion, a *p*-quinone-immonium derivative (Fig. 16*C*): This structure is supported by the fact that only mono-substituted hydroxylamine, semicarbazone, and *p*-nitro-phenylhydrazine derivatives could be obtained (Green and Richter 1937; Braconier, Le Bihan, and Beaudet 1943). The synthesis of adrenochrome through oxidation of adrenaline by silver oxide or in presence of a polyphenolase justifies, however, the assumption of its basic structure as that of an *o*-quinone. Depending on the reaction to be interpreted, one or the other formulation may seem to be preferable; in the present paper the *o*-quinone formulae will be used for adrenochrome and oxo-adrenochrome (VI) (Fig. 22).

* It is possible that a similar mechanism prevails in the controlled oxidation of adrenaline to adrenochrome with oxidizing agents such as iodine, ferricyanide, or metallic ions in higher than catalytic concentrations; the reaction proceeds quickly and without any accumulation of intermediates by oxidation of the phenolic groups to adrenaline-quinone and adrenochrome. Chaix, Morin, and Jezequel (1950) comment on the fact that in bicarbonate buffer the oxidation by equimolecular proportions of cupric ion stops at the adrenochrome level while it exceeds it in phosphate buffer. They suggest that a complex is formed between adrenochrome and the phosphate ion; and assume further that this complex formation, by virtue of interference with phosphorylations, is responsible for the inhibition of glucose metabolism in tissue by adrenochrome. The oxidation of adrenaline by equimolecular or higher concentrations of oxidizing agents has not been examined in detail in the present investigation, but it would seem that the observation is equally well explained by the fact that adrenaline dissolves the (basic) copper carbonate formed in bicarbonate buffer while it does not, or only to a small extent, react with the tertiary copper phosphate formed in phosphate buffer. The unstable phenolic salts of copper would therefore be quantitatively formed in bicarbonate buffer and decompose quickly to quinones. In phosphate buffer the concentration of the phenolic salt would be less and the *primary* oxidation rate would be lower, leading to an accumulation of intermediates, to the onset of destructive oxidation, and therefore to a higher overall consumption of oxygen. Figure 16 illustrates the result of one experiment. In the interpretation of the results it must also be considered that acidic compounds are formed in the oxidation of adrenaline; the gas exchanges recorded cannot therefore simply be taken as oxygen uptakes. Cf. also the observation that the auto-oxidation of adrenaline starts earlier in phosphate-free medium (Holtz and Kroneberg 1950).

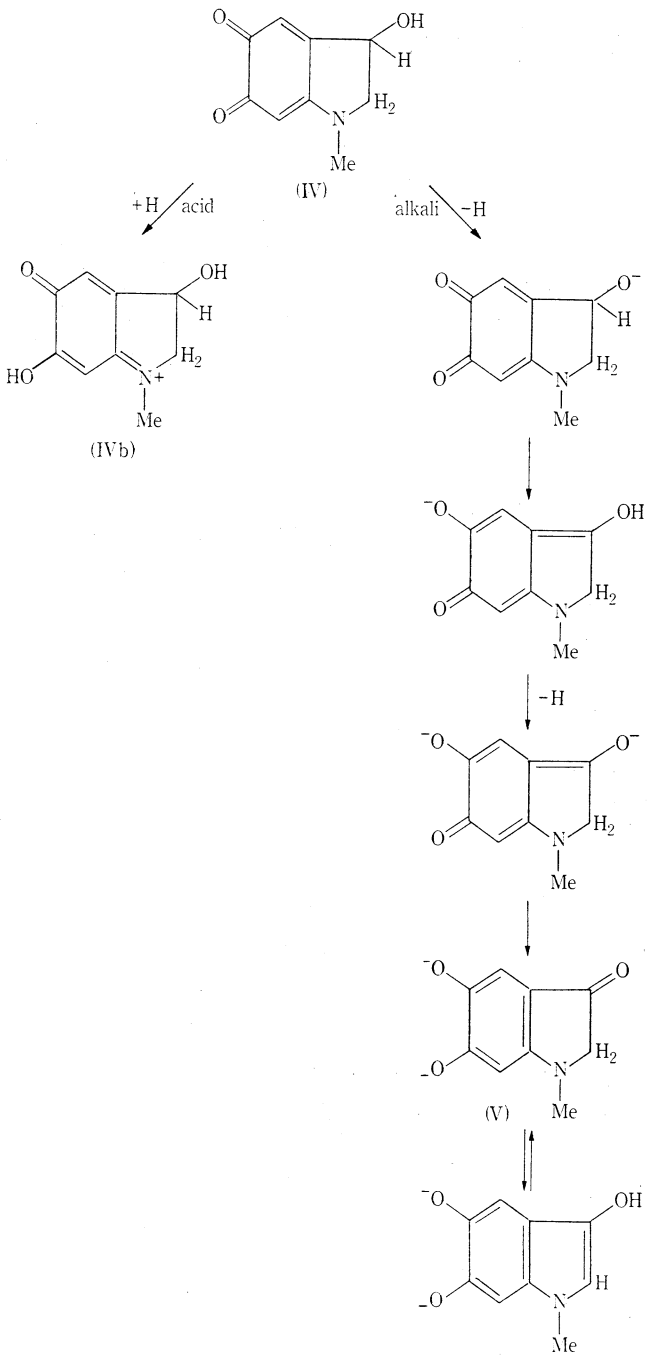

Fig. 15.—Formation of "green" and "fluorescent" adrenochrome by addition or loss of protons.

The following formulation is tentatively presented for the formation of "green adrenochrome" in acidic, and of "fluorescent adrenochrome" (*N*-methyl-5 : 6-dihydroxy-indoxyl) in alkaline medium. It is suggested that in acidic solution adrenochrome *adds one proton to a keto group of the o-quinone ring,* the positive charge of the proton being transferred to the nitrogen, forming

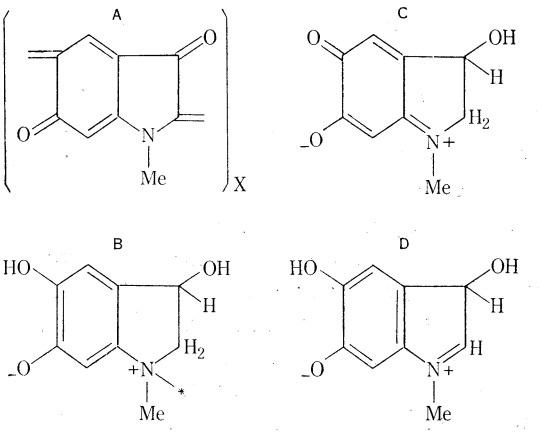

Fig. 16.—*A*: The structural element of melanin as proposed by Cohen (1945).
B: The semiquinone of the adrenochrome system as proposed by Harley-Mason (1948).
C: Zwitterion structure of adrenochrome.
D: Zwitterion structure of fluorescent compound as proposed by Harley-Mason (1948).

thus an immonium ion; only the structure in Figure 15 IV*b* is possible for this compound. Since the phenolic group cannot be supposed to be ionized in the acidic medium, the compound is not a zwitterion. It is suggested that in the acidic solution the remaining quinone oxygen reacts slowly with the $-CH_2-$ group activated by virtue of its position alpha to the immonium bond as a first step towards condensation. If the green colour observed is significant for this reaction, 30-60 minutes are required to complete it at room temperature. Neutralization at this stage no longer re-forms the red colour of adrenochrome. It is supposed that further oxidation takes place at the alcoholic group of the five-membered ring and that further condensation to chain structures occurs. On releasing the proton originally added, the positive charge on the nitrogen disappears and the condensation compound becomes insoluble, explaining the black precipitate formed within 24 hours.

In alkaline solution *adrenochrome loses two protons from the five-membered ring;* the negative charges thus created are transferred by electron rearrangement to the keto groups of the quinone ring, forming thus an ionized diphenol (Fig. 15 V). The transformation is almost instantaneous, and it is not reversible, since on neutralization or acidification the two protons lost are added *at the localization of the negative charges*: instead of adrenochrome the isomeric

4 : 5-dihydroxy-*N*-methyl-indoxyl is formed. On oxidation of the compound two electrons are removed from the ionized phenolic groups and oxo-adrenochrome is formed.

Since the successive removal of two protons first and then of two electrons constitutes only one oxidation step, the remarkable fact results that in the removal of two electrons and two protons from adrenochrome three distinct compounds figure in the reaction; the quinone adrenochrome, the diphenolic intermediate, and finally the keto quinone oxo-adrenochrome.

Melanin.—Bacq's statement, "The complete oxidation of adrenaline to melanin requires eight to nine atoms of oxygen per molecule of adrenaline and some CO_2 is evolved," citing Welsh (1934) as reference, is not correct. The analyses of the purest specimens of melanin indicate an oxidation level corresponding to the uptake of three atoms of oxygen, and the structure in Figure 16*A* has been proposed as the constituent unit of the molecule (Cohen 1945). This oxidation level is in agreement with the observation (in the trials of polyphenolase preparations) that the formation of a brown turbidity always indicated oxidation *beyond* the adrenochrome level even before the manometric readings could confirm it. The five to six more atoms of oxygen consumed by the solutions are therefore used in further and finally destructive oxidation of part of the adrenaline present. No conditions could be found that lead to a quantitative formation of melanin with an oxygen uptake of three atoms only; it would appear that melanin formation is a side reaction occurring during the slow destructive oxidation of adrenaline at physiological pH, and characteristic of it.

(*c*) *Fluorescence of Adrenaline Oxidation Products*

Adrenaline, especially in acidic solution, shows a slight fluorescence of its own. Loew (1918) was probably the first to describe the *strong* fluorescence observed during alkaline oxidation of adrenaline. West (1947), basing his work on that of Gaddum and Schild (1934) and others, presented an assay of adrenaline by the measurement of the maximum fluorescence obtained under standard conditions; Fischer and Lecomte (1949) used an almost identical procedure for the estimation of adrenochrome. Obviously the appearance of fluorescence is not specific for either substance. In fact, any adrenaline solution oxidizing at physiological pH gives, during the early stages of oxidation, fluorescence on alkalization, on alkaline oxidation, or on reduction. The present investigation is mainly concerned with the oxidation level of the fluorescent compounds and with the conditions under which red or fluorescent reaction mixtures are formed.

(i) *Fluorescence from Adrenaline*

At alkaline pH.—Figure 17 shows the fluorescence of M/2500 adrenaline with various concentrations of alkali under otherwise identical conditions. The maximum intensity of fluorescence is not obtained directly on admixture of the alkali; it develops slowly, especially with sodium carbonate or low concentration of free alkali. Carefully paralleled experiments suggest that the maximum

is reached not before the uptake of one atom of oxygen for one molecule of adrenaline and not later than corresponds to the uptake of two to three atoms. Then the intensity of fluorescence decreases and all fluorescence has disappeared when about six atoms of oxygen have been consumed.

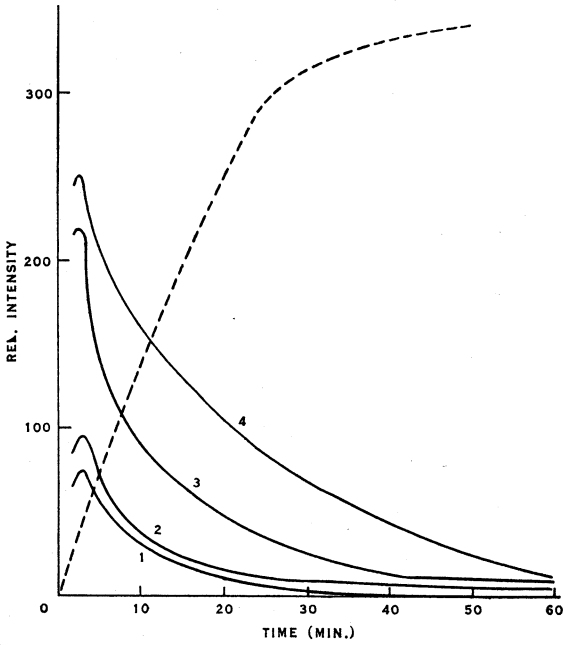

Fig. 17.—Adrenaline, fluorescence during alkaline oxidation. Final adrenaline concentration M/2500; 20°C.; tube frequently shaken to ensure adequate aeration.
Curve 1: Adrenaline in 0.5N sodium carbonate.
Curve 2: Adrenaline in 1.5N sodium carbonate.
Curve 3: Adrenaline in 0.5N caustic soda.
Curve 4: Adrenaline in 1.0N caustic soda.
The adrenaline oxidation curve in 1.0N caustic soda is superimposed in dots to illustrate the relation of fluorescence to oxygen uptake.

West (1947) proposed his assay for adrenaline concentrations of 10^{-6}-10^{-7}M, using sodium carbonate as the alkaline medium; the present authors could repeat his results. At higher adrenaline concentrations, however, it becomes difficult to maintain standard conditions since the intensity of the fluorescence depends not only on the HO ion concentration but also on the rate of aeration. If a solution of M/500 adrenaline, rendered alkaline with dilute sodium carbonate, is divided into three aliquots and air is excluded from the first by overlaying with paraffin oil, while the second is left standing and the third kept shaking, the fluorescence lasts longest in the first and least in the last tube; the peak intensity, however, is highest in the last and lowest in the first tube. It

is very doubtful whether the maximum intensities reached by different procedures are comparable and no attempt is made in the present work to draw any conclusions from slightly higher or slightly lower fluorescence intensities.

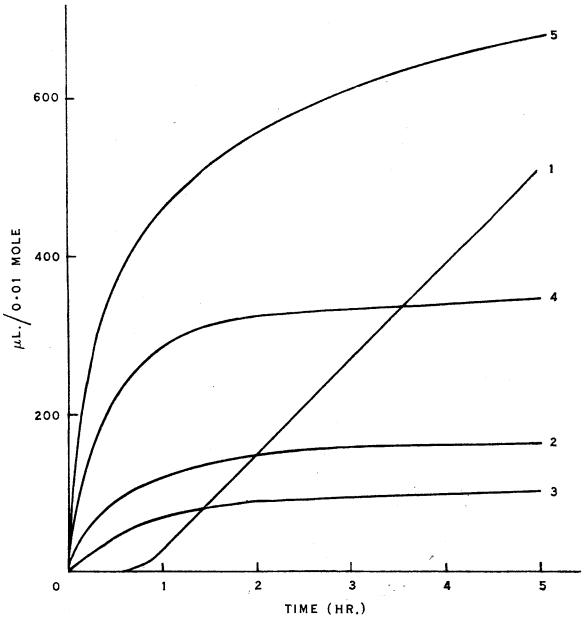

Fig. 18.—Adrenaline oxidation in the presence of sodium metabisulphite. Final adrenaline concentration M/2500, final sodium metabisulphite concentration M/2500, calculated as $Na_2S_2O_5$. M/15 phosphate buffer, pH 8.0; 37°C. The enzyme preparation showed no oxygen uptakes of its own. All reaction mixtures containing adrenaline and metabisulphate were colourless throughout the experiment but developed intensive green fluorescence.

Curve 1: Adrenaline alone.
Curve 2: Metabisulphite alone.
Curve 3: Adrenaline plus bisulphite.
Curve 4: Adrenaline plus 30 mg. enzyme and metabisulphite, pH 7.3.
Curve 5: Adrenaline plus 30 mg. enzyme, pH 7.3.

At physiological pH.—The auto-oxidation of adrenaline at physiological pH, catalysed or not, leads to coloured solutions. Fluorescent solutions are obtained only if the oxidation is conducted in the presence of a reducing agent.

If mixtures of adrenaline and sulphite are allowed to react in a Warburg manometer, the mixtures consume less oxygen than the sum of the two components singly reacted under the same conditions (Fig. 18); in the mixture the components show a "mutual inhibition of oxidation." At neutrality the rate of oxygen uptake is low and no visible changes may be observed for a con-

siderable time; at a slightly higher pH (8-9) *strongly fluorescent, colourless* solutions are obtained. The oxygen uptake quickly exceeds one atom of oxygen for one molecule of adrenaline but then the curve levels out and flattens between the levels of one and two atoms. The further oxidation proceeds very slowly and the intensity of fluorescence remains almost constant for a considerable time (cf. Shaw's (1938) adrenaline assay). The same result can be observed if adrenaline is allowed to oxidize slowly in the presence of glycine.

At more neutral pH the onset of adrenaline oxidation in the presence of sulphite can be accelerated by a polyphenolase. The reducing agent does not appear to affect the action of the enzyme seriously; an initial rapid uptake of slightly over two atoms for one molecule of adrenaline is observed, but then the curve flattens out exactly as in the previous case (Fig. 18, curve 4). The solutions remain throughout completely colourless and strongly fluorescent and at no time is the red colour of adrenochrome observable. The maximum intensity of fluorescence is reached with the uptake of slightly over two atoms of oxygen and remains almost unchanged for several hours.

(ii) *Fluorescence from Adrenochrome*

On reduction.—Adrenochrome (or any of the coloured solutions in the early stages of adrenaline oxidation) is readily reduced to fluorescent solutions; almost any form of reduction leads to some fluorescence, but, since adrenochrome is unstable at acidic or alkaline pH, the reaction is best conducted at about neutral pH. Ascorbic acid, sulphite, bisulphite, metabisulphite, and other hydrogen donors of low redox potential—but not thiosulphate—give almost immediate discoloration. If pure adrenochrome in concentrations of M/2000 or less is used, the resulting solution is absolutely colourless and strongly fluorescent (Fig. 20, curve 2); it is moderately stable in the presence of the reducing agent but begins to decompose if the latter is removed.

The reaction is reversible. Iodine in potassium iodide is a suitable reagent to remove excess of sulphite; if it is added drop by drop, the end point of the sulphite titration is reached when the brown drops of iodine become surrounded by a large red halo before their colour finally disappears. If more iodine is added, adrenochrome is re-formed; excess iodine may be removed by thiosulphate. If care is taken to keep the reaction mixture at neutral pH, the reduction and re-oxidation may be repeated two or three times before the solution becomes too dilute and side reactions remove the reactants. For purposes of physiological investigation of the fluorescent compound, dimercaptopropanol (BAL) is a good reducing agent for adrenochrome since in the concentrations needed neither BAL nor its oxidation products are physiologically active.

On alkaline oxidation.—Figure 19*A* shows the fluorescence measured on treating M/2500 adrenochrome with varying concentrations of alkali, and Figure 19*B* some results obtained with adrenochrome dilutions of 10^{-5}, the concentration used by West for his assay of adrenaline. Old or too concentrated adrenochrome solutions incline to form a fluorescence with a brown or orange tint, with

pure adrenochrome the solutions are *yellow* and identical with those obtained under the same conditions from adrenaline. The maximum intensities observed are slightly higher than those given by the same concentrations of adrenaline. The differences are: with adrenochrome the peak intensity is reached almost immediately on admixture, and the duration of the fluorescence is shorter than with adrenaline.

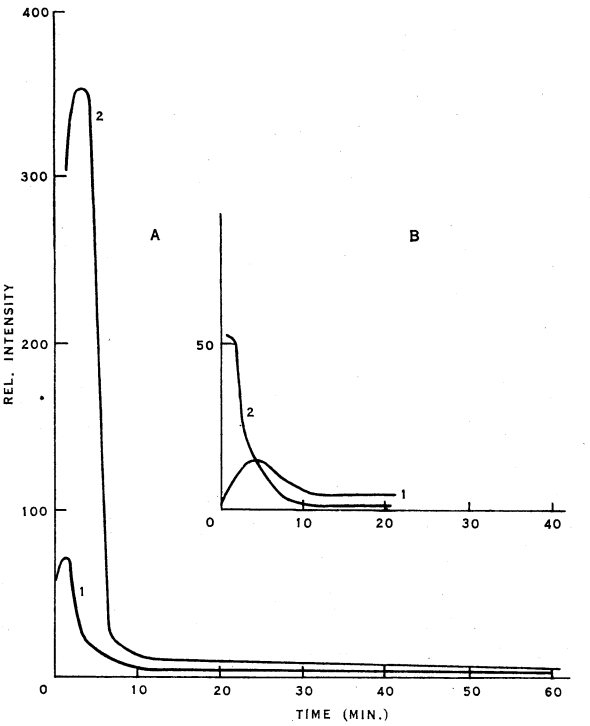

Fig. 19.—Adrenochrome, fluorescence during alkaline oxidation. *A*: Final adrenochrome concentration M/2500; 20°C.; tubes shaken to ensure adequate aeration.
Curve 1: Adrenochrome in sodium carbonate 1.5N.
Curve 2: Adrenochrome in 1N caustic soda.
B: Final adrenochrome concentration M/100,000.
Curve 1: Adrenochrome in 1.5N sodium carbonate.
Curve 2: Adrenochrome in 1N caustic soda.

The fluorescence formed on alkaline oxidation is of higher intensity than that formed on reduction. The addition of alkali to reduced adrenochrome further increases the fluorescence (Fig. 20, dotted line).

(iii) *Reactions of the Fluorescent Solutions*

The reactions given are the same as those described for the early yellow-fluorescent solutions obtained on the alkaline oxidation of adrenaline: positive

tests for the catechol grouping and for the indole or indoxyl structure. These reactions are given whether the fluorescence has been formed by reduction, oxidation, or, as with "fluorescent adrenochrome," without admission of air. The solutions are moderately stable at neutral or slightly acidic pH, but decompose on attempts at separation of the fluorescent compounds, as well as on prolonged standing. They oxidize faster than adrenaline. The oxidation to red quinones is not always possible if the solutions are aged; it would appear that further decomposition of the compounds takes place without the formation of quinones and melanins. The composition of these final yellow-brown solutions has not been investigated.

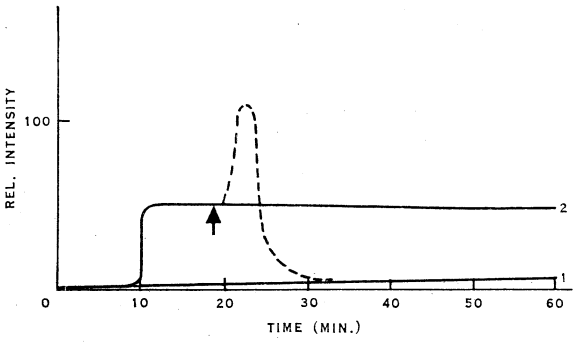

Fig. 20.—Adrenochrome, fluorescence with sodium sulphite. Final adrenochrome concentration M/2500; 20°C.; tubes shaken to ensure adequate aeration. Initial medium M/15 phosphate buffer, pH 7.0; sulphite concentration M/10.
Curve 1: Adrenochrome alone.
Curve 2: Adrenochrome plus sulphite; caustic soda, 1 ml. 1N, added at arrow.

IV. Conditions of Formation of Fluorescent or Red Solutions

(*a*) *At Weakly Alkaline pH*

If a dilute solution of adrenaline is rendered weakly alkaline by the addition of a few drops of N/10 KOH, it turns quickly red, consuming the oxygen dissolved in the liquid. After a few seconds, however, the colour fades and a yellow, fluorescent solution remains. On shaking with air, the red colour reappears, but fades again on standing. The procedure can be repeated three or four times, but then no more red colour is formed on shaking, nor is there any fluorescence left in the slightly yellow solution. The experiment, recorded by Loew (1918), is a modification of the repeated reduction and re-oxidation of adrenochrome just described, except that neither reducing nor oxidizing agents have been added. The oxidation of adrenaline to the red quinones is caused by more intensive aeration and the reduction of the quinones is brought about through the transfer of hydrogen to them from intermediates in the later stages of the reaction sequence. Figure 21 shows the fluorescence measured

during an experiment. It is seen that the intensity of fluorescence drops when the red compounds are formed on aeration, and increases again when they are reduced on standing, though not to the previous level, nor to the level of an identical solution that has not been shaken. After repeated shakings the stock of quickly reducible or oxidizable compounds becomes exhausted and the solutions no longer give catechol or indole reactions. The experiment succeeds equally well in barium hydroxide solution.

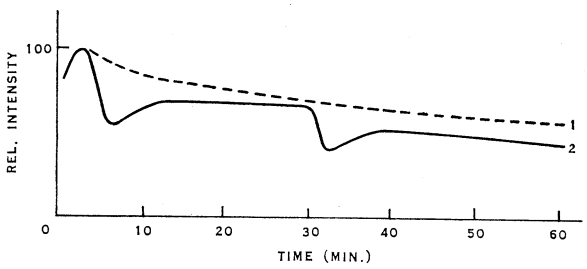

Fig. 21.—Adrenaline, fluorescence with different rates of aeration. Final adrenaline concentration M/2500; 20°C.

Curve 1: Adrenaline in 1.5N sodium carbonate, tube not shaken.

Curve 2: Same, but tube strongly shaken at 3 and 30 min.

A distinct pink colour was observed on shaking while the intensity of the fluorescence decreased, and disappeared when the fluorescence began to increase again.

(*b*) *At Physiological pH*

If adrenaline is allowed to oxidize under reduced oxygen supply, e.g. by overlaying the solution with a thin layer of paraffin, the formation of an early fluorescence is observed; but the reaction proceeds very slowly and the solutions soon become turbid and brownish. If the first steps of the reaction are accelerated, e.g. by placing a slice of dahlia tuber in the bottom of the test tube, a strongly fluorescent solution with a thin, red top layer is quickly obtained. On slight shaking, the whole liquid becomes temporarily red, but loses its colour again on standing. The final solutions are non-fluorescent, light brown, and slightly turbid, but considerably lighter in colour than the dark red-brown solutions obtained with intensive aeration. The oxygen uptakes under these conditions are difficult to follow.

(*c*) *Redox Potentials*

An attempt was made to determine the redox potential of the quinonoid system. Reliable values are difficult to obtain owing to the inherent instability of the system; the solutions undergo a continuous change towards the final destructive oxidation of adrenaline. The adrenaline/adrenaline-quinone potential, established by Ball and Chen (1933) by measurement at acidic pH, is taken

to be at neutrality only slightly lower than that of catechol/*o*-quinone, about + 0.38 volts referred to the normal hydrogen electrode. Falk (1949), quoting Wiesner (1942), places the leuco-adrenochrome/adrenochrome potential at about + 0.04 volts, slightly above that of methylene blue/leuco-methylene blue. Attempts were made to measure potentiometrically the redox potential of very pure adrenochrome solutions by treating them with increasing quantities of mild reducing agents like BAL or ascorbic acid. No complete titration curves could be obtained, but the readings at the start of the titration seem to indicate values about 0.1 volt below the potential of the quinhydrone electrode, between + 0.1 and + 0.2 volts. This value is higher than Falk's, but it appears to be in better agreement with observations made with leuco-methylene blue.

(*d*) *Action of Leuco-Methylene Blue*

Methylene blue itself, at physiological pH, has no quick action on either adrenaline or adrenochrome; the mixtures are blue with adrenaline, or violet with adrenochrome, and turn dirty violet or dirty brown as the atmospheric oxidation proceeds. If, however, a solution of adrenochrome, or any early red solution obtained on adrenaline oxidation, is treated with leuco-methylene blue, the red colour fades instantaneously, the mixture turns blue and strongly fluorescent, showing that *all quinones present have potentials sufficiently above that of methylene blue/leuco-methylene blue to allow a quick and quantitative reaction.* The further changes are slow at physiological pH. If the oxidation rate of the fluorescent compounds is accelerated by the addition of a few drops of N/10 KOH, the blue colour of methylene blue fades and a yellow-brown, *fluorescent* solution is formed that is slightly turbid through the precipitation of some leuco-methylene blue. The top layer in contact with air shows a thin but distinct blue zone; on shaking with air some methylene blue is re-formed but the colour fades again on standing. Under the conditions of the experiment methylene blue acts as oxidation mediator exactly as the quinone system in the experiments previously described. After 12-24 hours the blue colour of methylene blue begins to reappear and remains, though with low intensity since most of the dye has been decomposed. Methylene blue is reduced only while the oxidation of adrenaline proceeds at a noticeable rate, indicating the temporary presence in the oxidizing mixture of an effective concentration of compounds of a redox potential lower than that of methylene blue under the conditions. The final yellow solutions obtained on alkaline oxidation no longer react with methylene blue.

V. Discussion and Conclusions

(*a*) *The Redox Systems in Adrenaline Oxidation*

(i) *Nature of the Fluorescent Compounds.*—By their reactions the fluorescent compounds are to be considered as *o*-diphenols possessing indole or indoxyl structures, representing the reduction products of the corresponding red *o*-

quinones. Utevski (1944) and West (1947) proposed the fluorescent compound to be leuco-adrenochrome, corresponding to an oxygen uptake of *one atom of oxygen* for one molecule of adrenaline, or to the reduction product of adrenochrome formed by the uptake of two hydrogens and two electrons at the

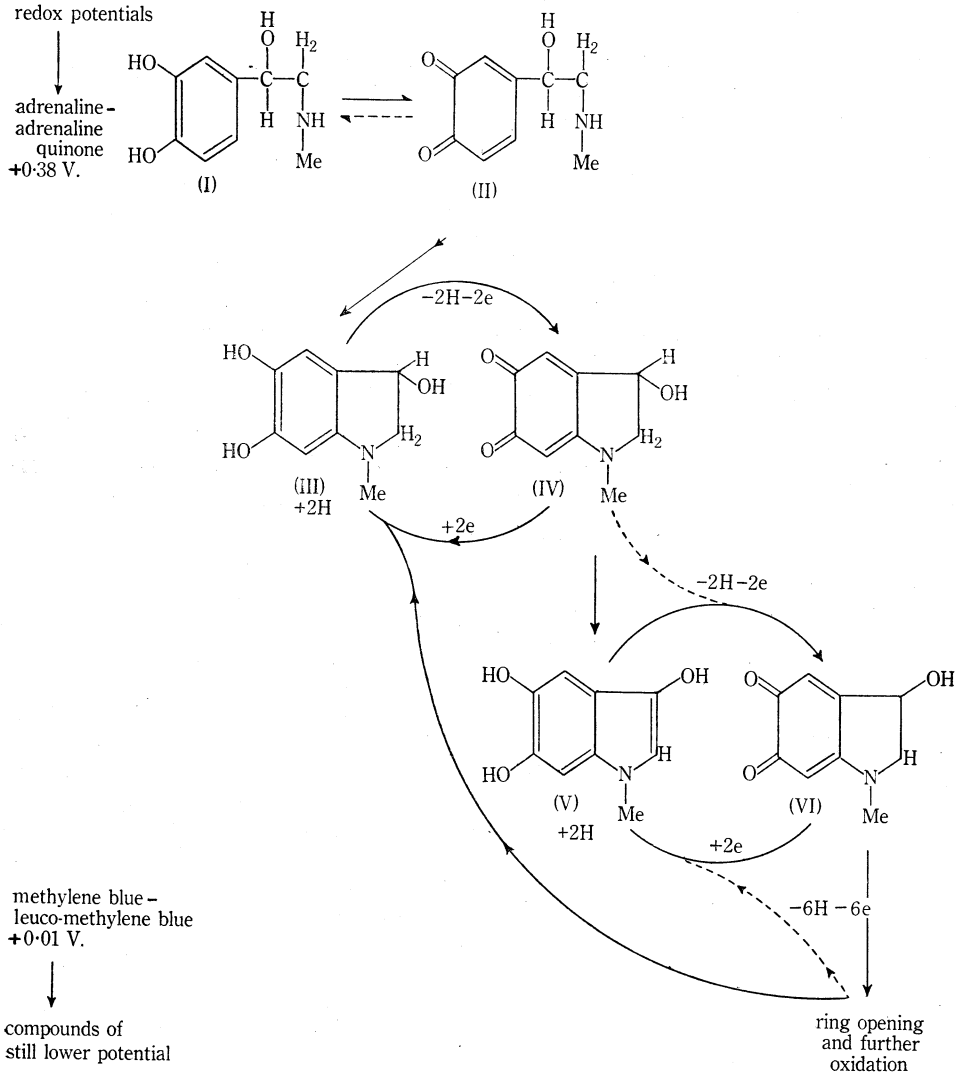

Fig. 22.—Redox systems in adrenaline oxidation.

quinone grouping. They state that the compound is fairly stable at pH 6-7, although decomposing on attempted isolation. Falk (1949) also considered leuco-adrenochrome to be the fluorescent compound, but assumed that it is rapidly oxidized by air at physiological pH. Harley-Mason (1948) claimed that leuco-adrenochrome is so unstable that it could not be formed during adrenaline oxidation since one should never obtain adrenochrome from it. He proposed

the fluorescent compound to be an isomer of adrenochrome, corresponding therefore to the uptake of *two atoms of oxygen* for one molecule of adrenaline and ascribed to it the zwitterion structure (Fig. 16*D*). Such an isomer has been obtained by Lund (1949) by controlled alkaline oxidation of adrenaline; the compound was separated in *yellow-green* crystals that gave fluorescent solutions and the catechol and indole reactions mentioned. Its composition was ascertained by analysis. In as yet unpublished work, Harley-Mason proposed the compound to be *N*-methyl-5 : 6-dihydroxy-indoxyl, the leuco form of oxo-adrenochrome (Fig. 22 VI) synthesized by Cohen (1945).

The present authors believe, however, that this compound is not the only fluorescent compound formed during adrenaline oxidation. They are of the opinion that the evidence presented by Harley-Mason proves merely the limited stability of leuco-adrenochrome, but does not disprove the possibility of its initial formation nor of its temporary role during the atmospheric oxidation of very dilute adrenaline solutions. The formation of *colourless* fluorescent solutions with oxygen uptakes of little over one atom for one molecule of adrenaline, and the reduction and re-oxidation of adrenochrome as described in this paper are accepted as demonstrating the existence of leuco-adrenochrome as a fluorescent oxidation intermediate. In the following discussion the fluorescent compounds as a group will be referred to as diphenols in relation to their oxidation products, the red quinones.

(ii) *Conditions of Formation of Fluorescent Di-phenols and of Red Quinones.*—It may be surprising that during the intensive oxygen uptakes of alkaline oxidation of adrenaline a very noticeable amount, if not the bulk of the reactants, is present in the *reduced* form as fluorescent diphenols, while during the slow oxidation at lower pH the bulk of the reactants is present in the form of the *oxidized* red quinones and their condensation product, melanin. Loew's experiment shows that in the same weakly alkaline solution fluorescent diphenols or red quinones can be observed *dependent on the rate of aeration.* It is suggested that the question of whether the solution contains the red quinones or the fluorescent diphenols depends on the relative rates of primary oxidation and of dehydrogenation. Under conditions of strongly alkaline oxidation the tendency of later oxidation products to transfer hydrogen to the quinones is so strong that only the reduced forms, namely the diphenols, are present, forming yellow, strongly fluorescent solutions; at lower pH the reactions of dehydrogenation proceed but slowly and the solution becomes enriched in the oxy forms, namely the red quinones, to a degree that even allows some condensation, red-brown, turbid, melanin-containing solutions being formed. If the intensity of hydrogen transfer is increased, e.g. by the addition of sulphite, glycine, or serum, fluorescent solutions are obtained.

These differences in the visible changes between oxidation at physiological and at more alkaline pH need therefore not necessarily indicate a fundamental difference in the mechanism of oxidation; in both cases the same end products

may be formed. At lower pH the red quinones accumulate in the solution, at higher pH they are rapidly dehydrogenated by the products of further oxidation.

(iii) *Redox Systems.*—It is suggested that the constant oxidation mechanism, indicated by the constant oxidation rate prevailing during the later phases of adrenaline oxidation at physiological pH, functions over redox systems that act as oxygen consumers and mediate the oxidation of later reaction products by accepting hydrogen and electrons. Owing to the instability of adrenaline-quinone it is unlikely that the adrenaline/adrenaline-quinone system plays a major role—with the possible exception considered in discussing the oxidation of adrenaline by a very pure polyphenolase. The first redox system formed would therefore be the leuco-adrenochrome/adrenochrome system. The functioning of adrenochrome as a hydrogen acceptor for malic and lactic dehydrogenases (Green and Richter 1937) proves the system to be fairly stable. Its action under physiological conditions has been suggested, e.g. for the synthesis of acetyl-choline in brain tissue (Torda and Wolff 1946) or in isolated intestine (Minz and Plotka 1947). It is considered that, during the early phases of adrenaline oxidation, leuco-adrenochrome is formed and acts as the reductant of the adrenochrome system; later in the reaction sequence the role may be shared or taken over by the *N*-methyl-5 : 6-dihydroxy-indoxyl/oxo-adrenochrome system. Figure 22 presents a scheme of the quinonoid systems together with such indications to redox potential as appear to be justified. It must be noted that:

(1) Although some of the later oxidation products may preferably transfer hydrogen to a suitable acceptor, quinone, or methylene blue, there is no evidence that the primary oxidation mechanism is confined to the re-oxidation of the diphenols. Some oxidation intermediates may react directly with oxygen;

(2) The visible changes observed during the oxidation of adrenaline show the presence of red quinones or of fluorescent diphenols; this observation does not exclude the functioning of other than quinonoid redox systems, say of indoxyl or dioxindol structures.

(*b*) *The Mechanism of Destructive Adrenaline Oxidation*

(i) *Onset of Oxidation*

Auto-catalysis and metal catalysis.—Adrenaline in an ideal pure solution at neutral pH would possibly be indefinitely stable; actually oxygen uptakes become noticeable *after a time lag* of several hours. It is accepted that the onset of oxidation is due to traces of metallic ions present in the solution (or, at more elevated pH, to HO ions). The action of these ions can be assumed to consist in the formation of unstable adrenaline ions, which readily lose electrons, adrenaline-quinone being formed in the process. The overall reaction is presented in Figure 23 I. The electrons liberated either reduce the metallic ions or, at higher pH, react directly with atmospheric oxygen. The adrenaline-quinone rearranges itself to leuco-adrenochrome, which in turn is oxidized by

the same mechanism, though at a faster rate, to adrenochrome. The latter accumulates in the solution which, towards the end of the time lag, acquires a faint pink tint. Higher concentrations of catalyst, metallic or HO ions, or higher temperature shorten the time lag.

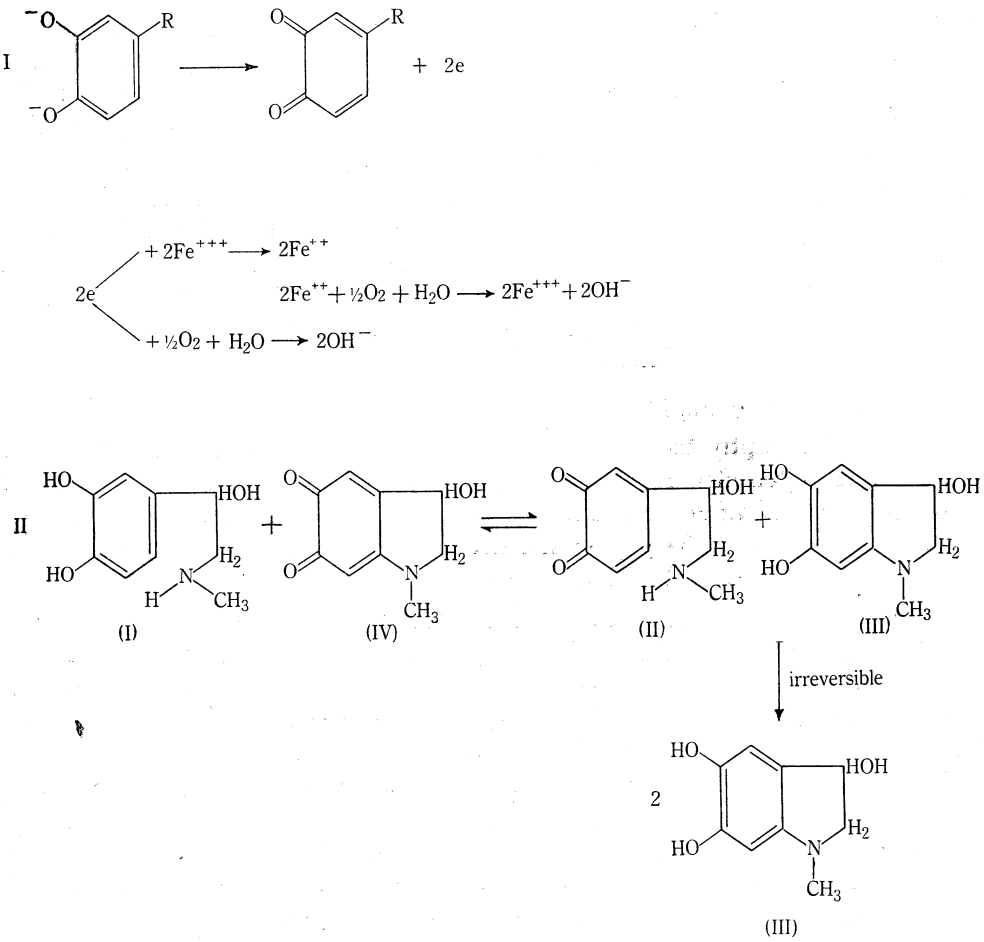

Fig. 23.—I. Mechanism of metal and HO ion catalysis.
II. Mechanism of adrenochrome catalysis.

Adrenochrome catalysis.—A different mechanism must be considered to explain the fact that the addition of small amounts of adrenochrome leads to exactly the same shortening of the time lag. It is suggested that the redox potentials of the adrenaline/adrenaline-quinone system and of the leuco-adrenochrome/adrenochrome system are near enough to each other for the reductant of higher potential, namely adrenaline, to be oxidized by the oxidant of lower potential, namely adrenochrome (Fig. 23 II from left to right); adrenaline-quinone and leuco-adrenochrome are formed in the process, or—owing to the quick rearrangement of the former—two molecules of leuco-adrenochrome. The

latter is oxidized at a faster rate than adrenaline and the sluggishly oxidizable adrenaline can be considered as being "mobilized" by the action of adrenochrome and in proportion to the adrenochrome concentration reached. Since adrenaline-quinone is short-lived, the redox potential of the adrenaline system will remain low, while that of the adrenochrome system remains high as long as the leuco-adrenochrome formed is oxidized at the rate of its formation. The solutions will contain unreacted adrenaline and adrenochrome without any effective concentrations of adrenaline-quinone or leuco-adrenochrome, i.e. of redox systems.

Chemical mechanism during the time lag.—It is suggested that the auto-oxidation of adrenaline at physiological pH is *started* by such traces of metallic ions as are present in the solution and then *further promoted* by adrenochrome catalysis. The oxidation rate remains low as long as the solutions contain adrenaline and adrenochrome alone, but the conditions change when the capacity of the primary oxidation system is reached and leuco-adrenochrome begins to accumulate in the solution.

(ii) *The Period of Rapid Acceleration*

Almost abruptly the oxygen uptakes begin to increase and the colour of the solution intensifies from pink to red. This period of acceleration is of short duration, about half an hour, and terminates in a steady, almost constant oxidation rate. It is tentatively proposed that the acceleration is due to the formation of more readily oxidizable compounds.

The onset of the acceleration.—The onset of acceleration is hastened by the addition of catalysts, but it is only little affected by the concentration of adrenaline (Fig. 3). It seems to coincide with the moment when in an unbuffered solution the pH begins to drop and appears to depend on the oxygen uptakes which, under the conditions of auto-oxidation, amount to about one-fourth atom of oxygen for one molecule of adrenaline. This uptake would correspond to the oxidation of about 5 per cent. of the adrenaline present to adrenochrome. Since the addition of this or a slightly higher proportion of adrenochrome just causes the time lag to disappear (Fig. 5, curve 3), it would seem that at these proportions the final oxidation mechanism is established without much delay.

The formation of more readily oxidizable compounds.—Indoxyl in alkaline solution is quickly oxidized by atmospheric oxygen. The oxygen substitution in the benzene ring will certainly not lower the oxidizability of the compound and it is likely that the formation of indoxyl structures is the critical step towards the further oxidation of adrenochrome. The formation of *N*-methyl-5 : 6-dihydroxy-indoxyl from adrenochrome in alkaline solution has been discussed. It is suggested that at physiological pH the transition takes place *in the mixture of leuco-adrenochrome and adrenochrome,* the overall reaction consisting of the oxidation of the dihydroindoxyl grouping of leuco-adrenochrome by the quinone grouping of adrenochrome. The mechanism of this step involves probably the disproportionation of free radicals. Oppenheimer and Stern (1939) reviewed evidence that the action of adrenochrome as hydrogen acceptor, e.g. in

the oxidation of amino acids, proceeds in a univalent reduction to a semiquinone. Harley-Mason (1948) found that in the reduction of adrenochrome by hydrogen in the presence of palladium-charcoal only one atom of hydrogen is consumed per molecule of adrenochrome and equimolecular proportions of *N*-methyl-5 : 6-dihydroxy-indole and *N*-methyl-5 : 6-dihydroxy-indoxyl were isolated from the reaction mixture. Harley-Mason proposed for the semiquinone formed the zwitterion structure in Figure 16 *B* with the unpaired electron at the positively charged nitrogen atom.

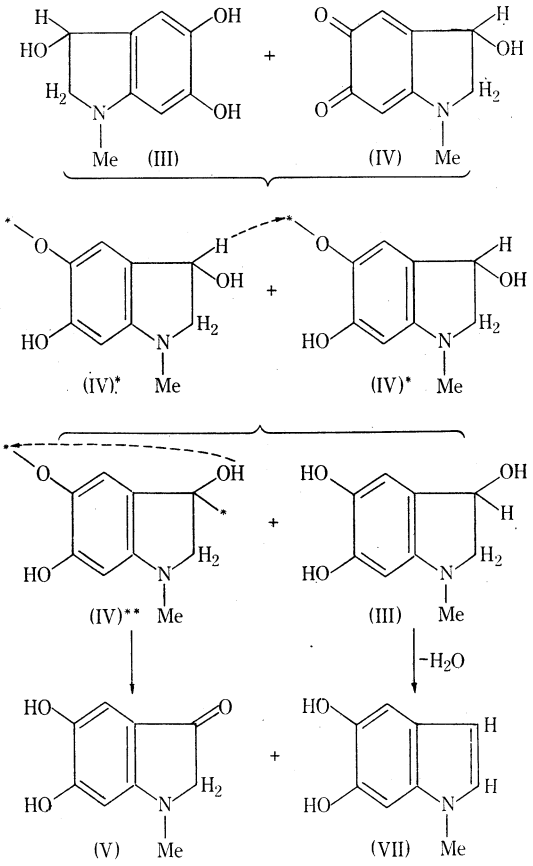

Fig. 24.—Free radical mechanism of the transition from the adrenochrome to the indoxyl structure (explanation in text).

A different formulation, for which the authors acknowledge the helpful advice of Dr. K. H. Pausacker,* and a tentative mechanism of the reaction sequence are presented in Figure 24. In the formation of the semiquinone (IV*) the hydrogen becomes attached to the 6 oxygen which, as shown by the possibility of a zwitterion structure for adrenochrome, has the higher electronegativity. The unpaired electron attaches itself to the remaining 7 oxygen.

* Department of Chemistry, University of Melbourne.

This free radical abstracts from the dihydroindoxyl grouping of another radical the 3 hydrogen atom, which is reactive through its position next to the HO group. In this disproportionation one molecule of leuco-adrenochrome is formed and one of the hypothetical highly unstable di-radical (IV**), which rearranges itself to the stable *N*-methyl-5 : 6-dihydroxy-indoxyl. It is supposed that under the conditions of auto-oxidation at physiological pH the leuco-adrenochrome formed returns into the adrenochrome redox system, while the indoxyl compound undergoes rapid further oxidation. The immediate effect of the reaction shows itself in increased oxygen uptakes leading to further oxidation and to the onset of destructive oxidation, with the formation of brown condensation products and dicarboxylic acids or other more acidic compounds. If the oxidation is slow, or on attempts at concentration and separation of the compounds further changes take place, e.g. condensations and other reactions, such as the dehydration of leuco-adrenochrome to *N*-methyl-5 : 6-dihydroxy-indole (VII).

(iii) *The Period of Constant Oxidation Rate*

The onset of constant oxidation.—The period of acceleration terminates under the conditions of atmospheric oxidation with the uptake of about half an atom of oxygen for one molecule of adrenaline, corresponding to the oxidation of about 12 per cent. of the adrenaline present to adrenochrome level.

(1) The role of the catalysts.—The oxidation rate finally established is the same within a wide range of catalyst concentrations (Fig. 4; also Falk 1949; Slater 1949, for haematin and methaemoglobin catalysis), and there is no evidence that the nature or amount of the catalyst plays any part in the course of the later oxidation. Da Fonseca-Ribeiro and Cardoso (1949) report that oxalic acid inhibits the auto-oxidation of adrenaline and suggested that salt formation between the acid and the metal ions competes with the formation of the phenolic salt. The observation could be confirmed and it is tentatively considered that early oxidation products after ring opening, possibly dicarboxylic acids, combine with the metallic ions, possibly by chelation, and prevent their further catalytic function (cf. also the inhibition of copper catalysis by complex formation with glycine (Verly 1948)). If this is the case, the reaction mechanism establishing and controlling the constant rate of oxidation would rest entirely between adrenaline and its oxidation products and be the same whichever way the period of acceleration was reached. This assumption is in agreement with the observation that adrenochrome catalysis also leads to the same final rate of oxidation (Fig. 5).

(2) The role of adrenaline concentration.—The constant rate of oxidation is maintained for several hours, from the time when about 80 per cent. of the adrenaline originally present must still be unreacted up to the uptake of 4-5 atoms of oxygen when at least 80 per cent. of the adrenaline must be accepted as being oxidized beyond opening of the ring structure. Evidently the later concentration of adrenaline is not one of the factors which regulate the oxidation rate. The final rate, however, appears to depend on the *original* concentration of adrenaline insofar as the same molar oxidation rate is reached (Fig. 3).

Maintenance of the oxidation rate.—On these observations it would appear that the mechanism of constant oxidation is *established* when a certain proportion of adrenaline to its oxidation products (corresponding to *c.* 12 per cent. adrenochrome) is reached, but that from then on adrenaline becomes merely the substrate of an oxidation mechanism that functions steadily, at full capacity and at a constant rate practically to the time when the stock of unreacted adrenaline becomes exhausted. It is suggested that two factors maintain the oxidation rate; the concentration and redox potential of the leuco-adrenochrome/adrenochrome system, and the retarding influence of later oxidation products.

(1) The redox potential.—The process of further and finally destructive oxidation proceeds in a series of steps following each other until the final products have no longer any efficient tendency to lose their hydrogen. Accepting adrenochrome as the critical level beyond which non-arrestible further oxidation starts, four to six atoms of oxygen are consumed by each molecule on its way. Even if only a fraction of this oxidation takes place over redox systems, such an intensive transfer of hydrogens must considerably increase the concentration of diphenols, mainly leuco-adrenochrome. This effect lowers the redox potential of the leuco-adrenochrome/adrenochrome systems and therefore decreases the rate of adrenaline mobilization on the mechanism suggested (Fig. 23 II from right to left); it leads also to a lowering of the concentration of the redox system which is depleted by destructive oxidation, but not replenished by adequate oxidation of adrenaline. The reaction mixtures in question are too turbid and complex to allow quantitative examination, and, without knowing the nature, redox potential, and reactivity of each intermediary it is impossible to outline the exact mechanism of the reaction sequence. It can, however, be understood that the reaction will stabilize itself at a state in which adrenaline enters the quinonoid systems at the same rate at which other molecules leave it.

(2) The role of later oxidation products.—In the presence of a moderate excess of metallic catalyst there occurs between the time lag and the period of final constant oxidation a period of steady, more rapid oxidation, the duration of which depends on the amount of catalyst added (Fig. 4). It is reasonable that excess of the catalyst constitutes a more efficient oxidizing system and leads to the rapid formation of a higher concentration of early oxidation products, mainly adrenochrome, and that the addition of adrenochrome itself has the same effect (Fig. 5). It is, however, difficult to see why the high oxidation rate, once established, should be of limited duration and eventually drop to about the same value that would have been reached without the addition of any catalyst. It is suggested that the solutions at this stage can be considered to be identical whichever way the high adrenochrome concentration has been affected: they contain *more adrenochrome* than would have been formed under the conditions of uncatalysed auto-oxidation and it would appear as if the excess adrenochrome forcibly formed were consumed before the "normal," final oxidation rate is established.

Ascorbic and pyruvic acids form mixtures with adrenaline that are very resistant towards oxidation. Obrecht (1939) proposed a mathematical formulation of the effect and Verly (1948) and Marquardt (1947) discussed it further. These mixtures consume less oxygen than the components singly, indicating that the tendency to react with atmospheric oxygen is considerably lowered. A late oxidation intermediary of adrenaline, keto acid, or other, may in the same way cause a decrease of "oxidizability" showing itself on all diphenols, but mainly on that of the highest potential, namely adrenaline. The effect would be that the bulk of adrenaline is preserved, and the components of the quinonoid systems are consumed until the concentration of the inhibiting intermediate, formed by a diminished amount of quinonoid systems, is low enough to allow further oxidation of adrenaline itself.

The sequence of acceleration and destructive oxidation.—If this presentation is accepted, there should be a *time interval* between the onset of the two reactions: First an increased oxidation rate is caused through the formation of indoxyl structures and their further oxidation to dioxindol and isatin structures; later, when the ring structures are opened, dicarboxylic acids are formed and inhibit the course of the earlier reaction in the manner discussed. Under suitable conditions, e.g. in the oxidation of adrenochrome alone, the first reaction, acceleration, overshoots the steady oxidation rate demanded by the conditions (Fig. 11); the final regulation is effected when, later on, the mechanism of destructive oxidation has become established. It is possible that the temporary increase of the early oxidation rate suggested in the oxidation of adrenaline-adrenochrome mixture (Figs. 5 and 12) represents also this phase of "overshooting," and it is suggested that the effect of excess of metallic catalyst (Figs. 4 and 14) is due to the same cause. In the oxidation of adrenaline alone this early "hump" cannot be expected since the concentration of adrenochrome increases so slowly that all mechanisms concerned can keep in step; the optimum oxidation rate is directly reached and then maintained.

(*c*) *Practical Applications*

(i) *Adrenaline Assay.*—West's assay of adrenaline (1947) by determination of the maximum intensity of the fluorescence formed under standard conditions of alkaline oxidation appears to be reliable for the dilutions proposed (10^{-6}-10^{-7}M) so long as it is ascertained that the unknown specimen contains adrenaline only. With higher concentrations af adrenaline the development of fluorescence depends too much on the conditions of the experiment to ensure reliably constant conditions. Furthermore, the fluorescence formed from early adrenaline oxidation products, viz. adrenochrome, is of the same order of intensity as that of adrenaline itself . The assay is not specific.

The colorimetric assay by measuring the adrenochrome colour formed on oxidation with iodine followed by destruction of the excess iodine with thiosulphate (Evans and Raper 1937) suffers from the same disadvantage since leuco-adrenochrome also is oxidized to adrenochrome by the same procedure. Its presence may, however, be detected by the fact that the solution shows a

noticeable fluorescence; adrenaline itself is only slightly fluorescent under the conditions. A further disadvantage is that the colour of adrenochrome is unstable, melanin condensation taking place very quickly.

The oxygen uptakes at physiological pH will only demonstrate the presence of a steadily functioning redox system; for estimation of the concentration of adrenaline or its lower oxidation products they are valueless, especially if other reductants, amino acids, etc., are present that may react with the system. The initial rapid oxygen uptakes at strongly alkaline oxidation (six atoms for one molecule of adrenaline, four for one of adrenochrome) measure possibly the oxygen required for the opening of the ring structures and may occasionally give confirmatory evidence as to the content of a mixed solution.

The formation of a strong fluorescence on anaerobic alkalization may reveal the presence of adrenochrome or other quinones, but it is doubtful whether it allows very accurate determination. The same applies to the formation of fluorescence by reduction.

It would appear that any of the methods mentioned will assay single compounds, but that in the assay of mixtures all of them together will have to be considered, combined with physiological methods for unreacted adrenaline and chemical methods for the determination of indol structures. The procedure is tedious and uncertain, since the mixtures undergo further changes while waiting for examination. It is hoped that the results presented will allow in some cases to predict what must and what cannot have happened in the solutions.

(ii) *Interpretation of Physiological Effects of Oxidizing Adrenaline Solutions or Adrenaline Oxidation Products.*—(1) If the effect of a single compound is to be investigated, synthetic oxidation intermediates will have to be chosen; adrenochrome, Lund's fluorescent adrenochrome isomer, the fluorescent reduction product obtained from adrenochrome with BAL, the fluorescent solution obtained by anaerobic alkalization of adrenochrome, and Cohen's oxo-adrenochrome. It is, however, doubtful how long even pure compounds will be stable in contact with metabolizing tissue or enzymes; in the test tube they begin to oxidize after a varying time lag. Probably the best that can be expected is that the reaction mixtures will not contain components of an oxidation level structurally lower than that of the starting material, say that starting from adrenochrome one will not have to count with adrenaline or adrenaline-quinone structures, or, starting from oxo-adrenochrome there will be no leuco-adrenochrome or adrenochrome.

(2) Only at the start of its oxidation will an adrenaline solution contain mainly adrenaline and some adrenochrome. Very soon the solution contains the components of the redox system and their condensation products besides decreasing amounts of adrenaline and increasing amounts of later oxidation products. The same will happen with oxidizing solutions of leuco-adrenochrome. These redox systems are very reactive and the physiological effect of such solutions will be due to individual and possibly specific effects of the components

as well as to the general interference of the redox systems with the metabolism of the tissue or other preparation. Especially delayed effects of an apparently "pure" intermediate, as for example recorded by West (1947) for leuco-adrenochrome, or prolonged tissue reactions must be suspected of being due not to the initial compound but to the formation of complex mixtures or redox systems during the further oxidation.

(3) On alkaline oxidation of dilute adrenaline, temporarily fluorescent, later yellow, but throughout clear solutions are obtained; at no time are noticeable concentrations of red quinones present and no melanin is formed. The same result is obtained at lower pH if excess of hydrogen donors, amino acids, or serum, are present in the reaction mixture; fluorescent solutions are obtained that turn slowly orange or brown, but no extensive melanin formation takes place. It appears to be likely that the oxidation of adrenaline under physiological conditions by non-specific oxidation mechanisms leads also to fluorescent compounds and their decomposition products but not to quinones and melanin, which latter is only formed if the quinones are allowed to accumulate in the solution. The same mechanism may apply to the oxidation of tyrosine, and will result if adrenochrome in moderate amounts is brought into contact with tissue or serum. Therefore the metabolism of adrenaline or any of its early oxidation products in the tissue cannot be accepted as proceeding on the same lines as the atmospheric oxidation in a buffer of the same pH under otherwise equal conditions. Particularly it cannot be accepted that melanin is the end product of the reaction, and the list of possible oxidation intermediates has to be extended beyond the adrenochrome stage. Owing to the high concentration of hydrogen donors, glucose metabolites, amino acids, etc., the quinonoid redox system is present mainly in the reduced form. This fact cannot be without effect on the other oxygen-consuming mechanisms present (cf. the effect of adrenochrome on glucose metabolism (Randall 1946; Wajzer 1946, 1947; Meyerhof and Randall 1948; Marquardt 1947). Regardless of the original composition of the adrenaline oxidation mixture it will very soon contain only insignificant amounts of quinones, and consist mainly of reduced compounds, fluorescent diphenols, and others, and these will determine the physiological effects.

The investigation is being continued in an attempt to localize the peroxidative step in adrenaline oxidation and to differentiate between the fluorescent solutions formed under different conditions.

VI. Acknowledgments

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