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OXIDATION OF *p*-PHENYLENEDIAMINE AND ADRENALINE IN ENZYMIC AND COPPER-CATALYSED REACTIONS

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THE study of the oxidation of amines by serum has taken two directions. One, involving the measurement of the oxygen consumed in reactions of this type, is characterized by the studies of the enzyme, ceruloplasmin (HOLMBERG and LAURELL, 1948, 1951*a*, *b*). The second, based upon colour formation, is mainly concerned with whole serum and the amines, *p*-phenylenediamine, N,N-dimethyl-*p*-phenylenediamine, and adrenaline (LEACH and HEATH, 1956; LEACH *et al.*, 1956; AKERFELDT, 1957*a*, *b*; ABOOD, 1957). Of late, these two lines have merged, with the suggestion that the colour forming component of serum could be ceruloplasmin (LEACH *et al.*, 1956).

Recent reports (subsequently disputed) that the blood of schizophrenics is high in oxidase activity (AKERFELDT, 1957*a*, *b*; ABOOD, 1957; BROWN, 1957; ANGEL *et al.*, 1957; ABOOD *et al.*, 1957) stimulated in this laboratory a study of the properties of ceruloplasmin. In the course of this investigation, findings contrary to some of the published results came to light and they are reported below.

MATERIALS AND METHODS

Serum. Human serum for testing was obtained by drawing 20 ml of blood from the antecubital vein, allowing it to clot, and centrifuging. Human serum for the isolation of ceruloplasmin was obtained by dialysing outdated blood bank serum to remove the citrate and dextrose. Retroplacental serum was obtained at the UCLA Medical Center from a patient in delivery. The blood, contaminated with amniotic fluids, was centrifuged.

Pig serum was obtained at the slaughterhouse: the blood was collected in a large stainless steel pail and allowed to clot during transportation to the laboratory (about 1 hr).

Ceruloplasmin. The procedure of HOLMBERG and LAURELL (1948) was followed with the exception that occasionally the chloroform–alcohol extraction was replaced by repeated ammonium sulphate fractionations at 42·5 to 47·5% saturation. Details of a typical preparation are given in Table 1. It can be calculated from the data of HOLMBERG and LAURELL (1948) that purified ceruloplasmin contains 21 μg Cu/mg N, so that our preparation still contained extraneous protein. Paper electrophoresis confirmed this calculation, for the final preparation, although apparently homogeneous at pH 8·6, showed two distinct bands at pH 7·4.

In order to follow the purification, an arbitrary unit was defined. The colorimetric estimation of oxidase activity reported by ABOOD *et al.* (1957), which employs *p*-phenylenediamine as substrate, was used. Test specimens were incubated for 0·5 hr rather than 1 hr as recommended by Abood as it was found that the ascorbic acid level had no effect with 0·5 hr incubation. One unit of activity was defined as that quantity which gave a change of optical density of 0·01 at 490 mμ when incubated

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at 37° for 0·5 hr with 0·5 ml of 0·1% *p*-phenylenediamine solution buffered at pH 6·8 with tris-hydroxymethylaminomethane (tris).

Substrates. *p*-Phenylenediamine was obtained from Distillation Products Industries; L-adrenaline bitartrate and L-noradrenaline were products of Winthrop-Stearns; DL-3:4-dihydroxyphenylalanine, serotonin creatinine sulphate, β-(3:4-dihydroxyphenyl)ethylamine (dopamine), tyramine hydrochloride, γ-aminobutyric acid, (GABA) and histamine diphosphate were Cfp grade products of the California Corporation for Biochemical Research. $CuSO_4 \cdot 5H_2O$ was Mallinckrodt AR grade.

Methods. Conventional Warburg methods were employed to measure oxygen uptake. Reactions were started by tilting substrates from the side arms of 20 ml Warburg flasks into the main compartment containing enzyme and buffer. In some runs copper sulphate solution was tilted from a second side arm at the same time. Both acid and alkali center wells were employed. In some experiments the system was flushed with oxygen before tilting.

Copper was determined by the method of GUBLER *et al.* (1952).

RESULTS

p-Phenylenediamine was readily oxidized in 0·1 M-acetate buffer, pH 6·0, by all the preparations used (Fig. 1). The most active ceruloplasmin fraction obtained (alcohol-chloroform extract, Table 1), although still quite impure, exhibited a rapid oxygen

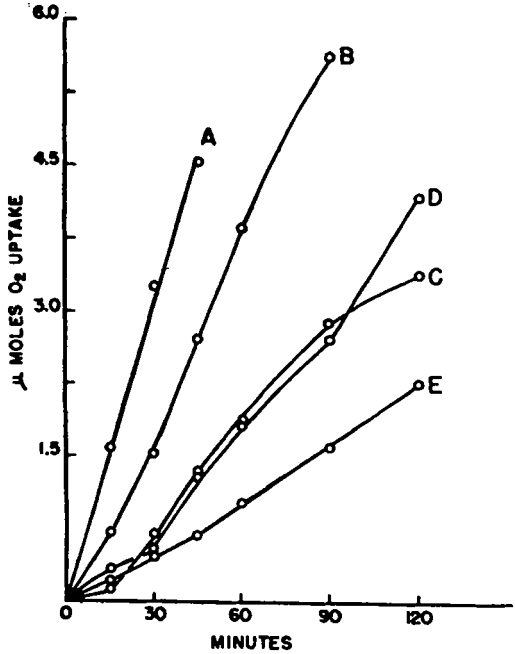

FIG. 1.—The ordinates represent μmoles oxygen taken up by a solution containing 0·5 ml of a *p*-phenylenediamine solution (10 μmoles), the solutions indicated below, and sufficient 0·1 M-tris buffer, pH 6·0, to make a final volume of 3·5 ml.

Curve A. 1·0 ml of alcohol–chloroform extract of pig serum (fraction 4a, Table 1).
Curve B. 1·0 ml retroplacental serum, after 24 hr dialysis against running tap water.
Curve C. 1·0 ml of alcohol fraction of blood bank serum (equivalent to fraction 3, Table 1).
Curve D. 1·0 ml of retroplacental serum.
Curve E. 1·0 ml of blood bank serum.

TABLE 1.—FRACTIONATION OF PIG SERUM PROTEINS

Fraction	Total units*	Units/mg N	μg Cu/mg N	Recovery of units (%)
1. Whole serum	$4{\cdot}42 \times 10^5$	14·2	0·11	—
2. 36–55%$(NH_4)_2SO_4$	$1{\cdot}55 \times 10^5$	46·1	0·35	35·2
3. 16% alcohol	$4{\cdot}96 \times 10^4$	65·4	0·79	11·3
4a. Alcohol–chloroform extract	$4{\cdot}21 \times 10^3$	532·8	3·52	0·9
4b. 42·5–47·5% $(NH_4)_2SO_4$†	$7{\cdot}86 \times 10^3$	364·3	3·06	1·7

* One unit is defined as that quantity of oxidase activity which gives a change of optical density of 0·01 at 490 mμ when incubated at 37° for 0·5 hr with 0·5 ml of 0·1% *p*-phenylenediamine solution buffered at pH 6·8 with tris.

† This fractionation procedure was repeated three times.

uptake with *p*-phenylenediamine (Curve A, Fig. 1). The preparation was clearly similar to that of HOLMBERG and LAURELL (1948); it had a deep blue colour and migrated as an α-2-globulin in an electrophoretic field. At pH 6·0, the pH at which maximal activity was reached, the K_m of the reaction of this fraction with *p*-phenylenediamine was $1{\cdot}9 \times 10^{-3}$ moles/litre, in good agreement with the reported value of $2{\cdot}5 \times 10^{-3}$ (HOLMBERG and LAURELL, 1951*a*). In spite of the apparent similarity of the two preparations, DOPA and adrenaline did not appear to be substrates for the enzyme, as had been reported (HOLMBERG and LAURELL, 1951*a*). Similarly, no oxygen uptake was found with serotonin, dopamine, tyramine, GABA, or histamine as substrates and the various serum preparations listed above as the source of enzyme activity, as has been reported (MARTEN *et al.*, 1958; PORTER *et al.*, 1957). A possible explanation of these findings might lie in the fact that in most of the studies in which oxidation with these amines was reported either dilute buffers (MARTEN *et al.*, 1958; PORTER *et al.*, 1957) were used or no buffer at all (HOLMBERG and LAURELL, 1951*a*).

The oxidation of adrenaline was then investigated. At pH 6·0 a $2{\cdot}9 \times 10^{-3}$ M-adrenaline solution in 0·04 M-tris-HCl buffer remained unchanged for long periods of time. After three hours at 35° no oxygen uptake was observed and only the faintest trace of yellowing was noted. Similar results were obtained in 0·1 M-tris-HCl buffer. If $CuSO_4$ was added to the buffer solutions to a concentration of 10^{-5} M, a rapid oxygen uptake was observed with the dilute buffer, but not with the more concentrated one (Fig. 2). Colour formation paralleled the oxygen uptake. At the conclusion of the run the pH had become alkaline in the experimental flasks (pH range 7·1–7·7) containing the 0·04 M-buffer, but remained at pH 6·0 in the controls to which no copper had been added and in the flasks containing the 0·1 M-buffer. When serum or serum preparations were added to these solutions (at pH 6·0) no oxygen uptake was found but a considerable amount of red colour appeared.

At pH 7·4, a $2{\cdot}9 \times 10^{-3}$ M-adrenaline solution in either 0·04 M- or 0·1 M-tris-HCl buffer took up some oxygen and developed a pink colour (Fig. 3). When the solutions also contained 10^{-5} M-$CuSO_4$, oxygen uptake was rapid and deep red colours developed. If, instead of $CuSO_4$, serum or enzyme preparation was added to the adrenaline-buffer solutions, a small increase in oxygen uptake above that measured

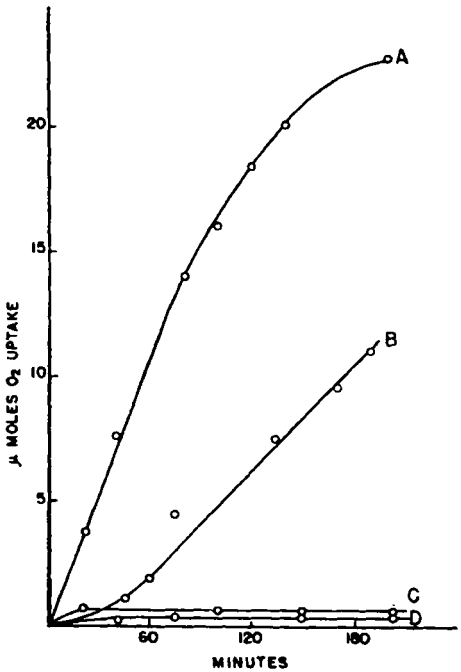

FIG. 2.—The ordinates represent μmoles oxygen taken up by a flask containing 0·5 ml of adrenaline solution (10 μmoles), $3{\cdot}5 \times 10^{-3}$ μmoles of $CuSO_4$, and the solutions indicated below:

Curve A. Sufficient 0·04 M-tris buffer, pH 6·0, to make a final volume of 3·5 ml.
Curve B. As A, but with 0·5 ml of pig serum added.
Curve C. As A, but with 0·5 ml of bovine albumin solution (3·50 g/100 ml) added.
Curve D. Sufficient 0·1 M-tris buffer, pH 6·0 to make a final volume of 3·5 ml.

with the buffer alone was seen, and a considerable amount of colour was formed. When, however, serum was added to the solutions which contained copper, colour formation and oxygen uptake were markedly diminished. Albumin also inhibited this reaction, but to a lesser extent (Fig. 3).

DISCUSSION

A summary of the results is presented in Table 2. At pH 7·4 no additions were needed to induce colour formation or oxygen uptake. This was most probably caused by trace metal impurities in the solutions. It has been observed previously (CHAIX *et al.*, 1950; VARÈNE, 1957*a*) that this trace metal catalysis is pH dependent and no effect is found at pH 6·0. The addition of copper to the solution at pH 6·0 induced the formation of some colour. This was probably caused by the formation of the copper-adrenaline complex demonstrated by CHAIX *et al.* (1950). The rate of the further reaction of this complex with oxygen is many times greater at pH 7·4 than at pH 6·0 (CHAIX *et al.*, 1950; VARÈNE, 1957*a*) so that vigorous oxygen uptake was

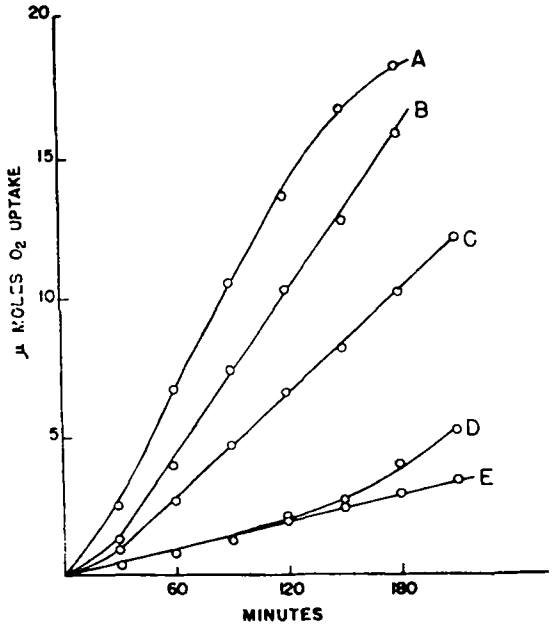

FIG. 3.—The ordinates represent μmoles of oxygen taken up by a flask containing 0·5 ml of adrenaline solution (10 μmoles), $3{\cdot}5 \times 10^{-3}$ μmoles of $CuSO_4$ (except E), and the solutions indicated below:

Curve A. Sufficient 0·1 M-tris buffer, pH 7·4, to make a final volume of 3·5 ml.
Curve B. As A, but with 0·5 ml of bovine albumin solution (3·50 g/100 ml) added.
Curve C. As A, but with 0·5 ml of alcohol fraction of pig serum (Fraction 3, Table 1) added.
Curve D. As A, but with 0·5 ml of pig serum added.
Curve E. As A, but containing no $CuSO_4$.

seen at the alkaline pH. Along with the oxygen uptake, the appearance of the red colour characteristic of adrenochrome (BOUVET, 1949) was observed. However, at pH 6·0 no further reaction of the complex was seen. When serum (or enzyme preparations, or albumin) was added to the system at pH 7·4 an inhibition of the copper catalysed oxidation was seen. This has been observed before (VARÈNE, 1957*b*; WALAAS and JERVELL, 1958) and it is generally considered to result from the binding of copper by protein. When, however, serum was added to the system at pH 6·0 an acceleration of the colour production, but not of oxygen uptake, resulted. This was probably due to a coupled reaction between the adrenaline-copper complex and some reducible component of serum. The suggestion that this coupling is enzymic is unnecessary; a copper-catalysed electron transfer could proceed non-enzymically. The fact that heat-treated serum has lost this ability to activate (PAYZA and HOFFER, personal communication) does not necessarily imply an enzyme, because heat coagulation will effect other components in serum as well as proteins.

The colour which appeared at pH 6·0 when serum and adrenaline were mixed without copper can be explained by the copper present in the serum and the trace

TABLE 2.—SUMMARY OF RESULTS ON THE OXIDATION OF ADRENALINE

Solution	pH 6·0		pH 7·4	
	Colour	O_2 uptake	Colour	O_2 uptake
Buffer A or B	None	None	Pink	*
Buffer B + $CuSO_4$	Yellow-pink	None	Dark Red	‡
Buffer A + $CuSO_4$	Red	‡	Dark Red	‡
Buffer B + Serum	Faint pink	None	Red	†
Buffer B + Serum + $CuSO_4$	Red	None	Dark Red	*

Flasks contained 0·5 ml. of adrenaline solution at the appropriate pH (10 μmoles/0·5 ml), 0·5 ml serum where indicated, 0·2 ml $CuSO_4$ solution at the appropriate pH (1·75 μmoles/ml) where indicated, and sufficient buffer A or B to make a total of 3·5 ml. Buffer A was 0·04 M-tris-HCl, pH 6·0 or pH 7·4 (as indicated). Buffer B was 0·1 M-tris-HCl, pH 6·0 pr pH 7·4 (as indicated).

* 0 to 3 μmoles oxygen uptake in 2 hr incubation at 35°.
† 3 to 7·5 μmoles oxygen uptake in 2 hr incubation at 35°.
‡ over 7·5 μmoles oxygen uptake in 2 hr incubation at 35°.

amounts possibly present in the water used. This amount of copper (the concentration of copper in serum is about $1{\cdot}6 \times 10^{-5}$ M, much of it associated with proteins) is sufficient to cause a rapid reaction at pH 7·4, and serum alone will produce colour in adrenaline solutions at pH 7·4. However, in this case oxygen uptake is considerably less than expected, possibly due to the presence of an electron acceptor competing with atmospheric oxygen.

Colour formation in these systems is, as has frequently been indicated, the result of a complex series of reactions. Initiated by metal activation of the amine (involving an enzyme or not), the reaction proceeds to highly polymerized, structurally unidentified compounds at unknown oxidation levels. There appears to be no true correlation between the intensity of the colour produced in these reactions and the extent of the oxidation.

SUMMARY

1. Oxidation of *p*-phenylenediamine was catalysed by serum and ceruloplasmin preparations. The optimal pH was 6·0 and the K_m of the reaction was $1{\cdot}9 \times 10^{-3}$.

2. At pH 6·0 neither serum nor ceruloplasmin preparations showed measurable oxygen uptake with adrenaline, noradrenaline, serotonin, dopamine, tyrosine, GABA, or histamine. Colour formation was, however, frequently noted.

3. When 10^{-5} M-cupric ion was added to the buffers, rapid acceleration of oxygen uptake was found with adrenaline at pH 7·4. When cupric ions were added to buffer of pH 6·0 no uptake was observed unless dilute buffers were used. In these experiments, the pH changed during the course of the run. When the pH became alkaline, oxygen uptake was observed.

4. Ceruloplasmin, serum, and bovine albumin inhibited the copper-catalysed oxidation of adrenaline at pH 7·4. Serum induced the appearance of colours at pH 6·0.

5. It is suggested that serum does not oxidize adrenaline enzymically and that

the observed oxygen uptake derives from metal-catalysed autoxidation at alkaline pHs.

6. Colour formation is not a reliable indicator of enzymic oxidation in these systems.

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