the presence of oxygen may occur spontaneously also. Nevertheless, the reduced coloured compound is capable of uniting with the enzyme surface (p. 633), where its reoxidation appears to be accelerated.

SUMMARY

1. From *Atropa belladonna* leaves a catechol oxidase has been isolated which is highly active in an insoluble form. It was obtained free from polyphenols and amino-acids.

2. It was found to oxidize catechol with the rapid uptake of approximately 2 atoms of oxygen/mol. catechol; further oxygen uptake was slow.

3. Phloroglucinol, *p*-cresol, adrenaline, *N*-methyladrenaline and aesculetin were also oxidized rapidly; hydroquinone and gallic acid more slowly; aesculin very slowly and resorcinol not at all.

4. Variation of pH between 4·5 and 8·3 had little effect on the rate of oxidation, cyanide inhibited very slightly; but diethyldithiocarbamate more strongly. Carbon monoxide inhibited, and the inhibition was not reversible by light.

5. Secondary oxidation of glycine took place with release of ammonia and uptake of additional oxygen. The volume of ammonia produced was approximately double the volume of extra oxygen consumed. Glyoxylic acid was isolated from the reaction products. No carbon dioxide was released. Pyruvic acid was similarly obtained from alanine, but the reaction was much slower than that with glycine.

6. Other amino-acids and dipeptides were oxidized much more slowly than glycine, and some not at all.

7. Formation of a red colour preceded the secondary oxidation. Once it is formed, its reduction by the amino-acid is non-enzymic, and its reoxidation in the presence of oxygen may occur spontaneously. Nevertheless, the reduced coloured complex is capable of uniting with the enzyme surface, and its reoxidation may be accelerated. Reasons are given for supposing that the coloured complex, probably a *p*-amino-*o*-quinone, is the immediate oxidizer of the amino-acid, and that the role of the polyphenolase is limited to its formation and possibly also its regeneration in the system.

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The Behaviour of Secondary and Tertiary Amines in the Presence of Catechol and Belladonna Catechol Oxidase

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The results presented in the preceding paper (James, Roberts, Beevers & de Kock, 1948) showed that a polyphenol oxidase from belladonna, using catechol as an intermediate, was capable of oxidizing certain primary amino-acids. The present paper describes the effects of the same enzyme system on secondary and tertiary amines, and, in addition, the oxidation of glycine which occurs when it is added to a complex of catechol and secondary amine in the presence of the enzyme.

EXPERIMENTAL

The enzyme was prepared as described in the preceding paper (James *et al.* 1948) and the manometric technique was essentially the same; the experiments were carried out at 30°, and the solutions were buffered to pH 7·8 by the addition of 0·1 M-phosphate mixtures. When two reactants were to be added separately to the main mixture in the manometer vessels, Keilin cups were employed.

RESULTS

The reactions of secondary amines with the enzyme-catechol system

No O_2 uptake was observed when secondary amines, e.g. dimethylamine, were added alone to an enzyme suspension and no colours developed. When catechol was also added a strong colour quickly appeared. It was noticed that, with secondary amines, this colour was different from that developed with primary amines and amino-acids. Thus, whereas methylamine, glycine, alanine, leucine, aspartic acid, glutamic acid and other primary amino-acids produced reddish-brown colours, the secondary amines, dimethylamine, proline, sarcosine and nortropine gave rise to deep royal-purple colours. Although the colours produced were different, it was clear that the secondary amines were reacting with an oxidation product of catechol in a way analogous to that which occurs with amino-acids, and manometric experiments were carried out to discover if any oxidation took place under these conditions.

Solutions of the desired amine (0·5 ml.; 0·1 M) were placed in the main chamber of manometer vessels with 50 mg. enzyme suspended in 1·5 ml. 0·066 M-phosphate buffer at pH 7·8, and 0·01 M-catechol (0·5 ml.) was placed in the side arms. Control runs with water instead of secondary amine were set up. After 15 min. shaking at 30°, the catechol was tipped in and the O_2 uptakes measured over 200–240 min.

Fig. 1 *A* and *B* shows the results obtained with the secondary amines listed above. In the controls, the catechol was oxidized vigorously in the usual manner, and the O_2 uptakes, which were very steep at first, flattened out as the substrate was used up. On addition of the secondary amine there was no significant increase in the rate of O_2 uptake, even after a period of 4 hr. Although royal-purple colours were developed in the presence of the secondary amines, it was obvious that the formation of the coloured complex did not involve any extra O_2 uptake, and, furthermore, there appeared to be no oxidation of the secondary amines under conditions in which amino-acids are oxidized.

The reactions of tertiary amines with the enzyme-catechol system

As differences in behaviour of amino-acids and secondary amines had been encountered, some experiments with tertiary amines were carried out. In particular, it was of interest to know whether the belladonna enzyme would oxidize the belladonna alkaloid, *laevo*-hyoscyamine, and the basic alcohol, tropine, of which it is the tropyl ester. Similar experiments to those described above for secondary amines showed that no colours were developed when catechol was oxidized by the enzyme in the presence of *laevo*-hyoscyamine, tropine or trimethylamine neutralized with hydrochloric acid. Manometric determinations established that the tertiary amines were not themselves oxidized (Fig. 1*C*).

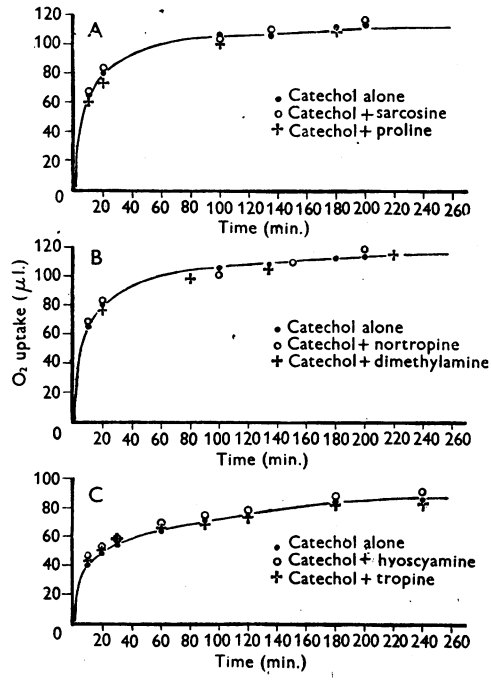

Fig. 1. Oxygen consumed by belladonna polyphenolase-catechol system; *A* and *B* with secondary amines and *C* with tertiary amines, all present in a concentration 10 times that of the catechol concentration (0·002 M).

The effect of addition of glycine to mixtures containing enzyme and equimolar concentrations of catechol and secondary amine

Reasons were given by James *et al.* (1948) for supposing that the formation of a coloured complex made up of 1 mol. of oxidized catechol and 1 mol. of amino-acid is an intermediate in the oxidation of amino-acids by this enzyme system. Since secondary amines may be used as substitutes for amino-acids in the formation of a coloured compound, the coloured compounds which they form might then act as intermediates in the oxidation of amino-acids. The experiments which follow show that this is in fact true.

The results of James *et al.* (1948) showed that even in the presence of a tenfold excess of secondary amine, no extra O_2 uptake occurred apart from that which was found with catechol only.

Manometric experiments were performed in which 0·05 M-catechol (0·1 ml.) was added from a Keilin cup to the main chambers of manometer vessels containing 50 mg. enzyme in 1·8 ml. buffer pH 7·8 and 0·05 M-secondary amine (0·1 ml.). Glycine (0·5 ml., 0·1 M) was placed in the side

arms of some of the vessels, and it was thus possible to begin the oxidation of the catechol by dislodging the Keilin cups, and then to add the amino-acid after any desired interval.

The oxidation of glycine in the presence of dimethylamine. Fig. 2 shows the results of experiments in which glycine was added after 20 min. (*A*) and 75 min. (*B*) to flasks containing (i) enzyme, catechol and dimethylamine (curves *a* and *b*); (ii) enzyme and catechol without dimethylamine (curves *c* and *d*).

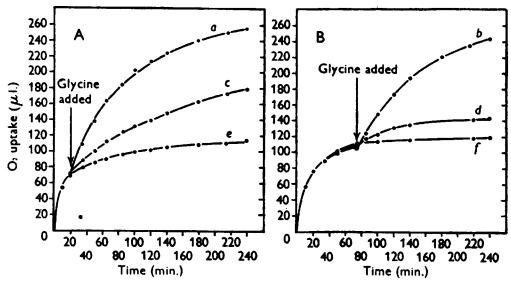

Fig. 2. The effect on O_2 uptake of adding glycine after *A*, 20 min. and *B*, 75 min. to mixtures containing enzyme, catechol and dimethylamine (*a* and *b*), and enzyme and catechol without dimethylamine (*c* and *d*). Curves *e* and *f* are controls to which no glycine was added.

Curves *e* and *f* show the O_2 uptakes of control flasks containing enzyme with catechol and dimethylamine to which no glycine was added; and in these the O_2 uptake stopped when 2 atoms O_2/mol. catechol had been absorbed. The O_2 uptakes of systems *a*, *c* and *e* (Fig. 2*A*) were almost indistinguishable from each other during the first 20 min., although purple colours had developed in *a* and *e*. At the end of this time, the glycine was tipped from the side arms of vessels *a* and *c*. A vigorous additional O_2 uptake then commenced in *a*, while in *c* also the glycine was oxidized, but at a noticeably slower rate. After 4 hr., i.e. 220 min. after the addition of the glycine, the oxidation in the presence of dimethylamine had accounted for a total additional uptake of 145 μl. O_2, while in its absence only 70 μl. had been absorbed. In Fig. 2*B* the O_2 uptakes of the three systems *b*, *d* and *f* were very similar until the glycine was tipped in after 75 min., and, by this time, the enzyme had almost completely oxidized the added catechol. In this case also, the addition of the glycine to the flask containing dimethylamine produced an immediate extra O_2 uptake (curve *b*) and 165 min. later the total amount of O_2 uptake due to the added glycine was 130 μl. The contrast with curve *d* (no dimethylamine), was even more marked than after 20 min., since the addition of glycine produced only a small additional uptake of 25 μl.

The O_2 uptake curves in Fig. 2, and a comparison of the amounts of glycine oxidation show clearly that glycine is oxidized much less readily when its addition, in the absence of dimethylamine, is delayed until after oxidation of catechol has begun, and, if this has progressed nearly to completion, the amount of glycine oxidation is very small indeed. When dimethylamine is present at the outset, there is no such diminution in the rates of oxidation when the addition of glycine is delayed to successively later stages. Indeed, when glycine was added after 75 min., the oxidation was slightly more rapid than that after 20 min., since almost equal amounts of glycine had been oxidized in the two cases at the end of 4 hr.

The oxidation of glycine in the presence of sarcosine and proline. It was found that sarcosine and proline, which are not themselves oxidized under the experimental conditions, acted in the same way as dimethylamine. When glycine was added after 100 min.

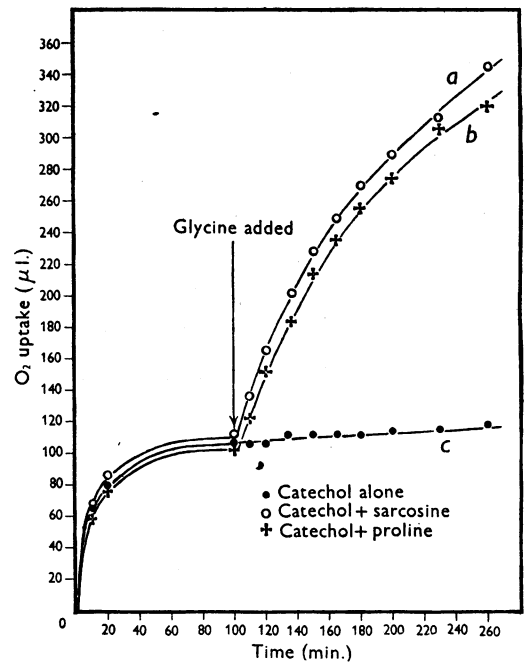

Fig. 3. The effect on O_2 uptake of adding glycine after 100 min. to (*a*) enzyme, catechol and sarcosine, and (*b*) enzyme, catechol and proline. Curve (*c*) is a control without added glycine.

to flasks containing sarcosine or proline, in addition to catechol and enzyme (curves *a* and *b* in Fig. 3), a rapid O_2 uptake began in each case. The oxidation of the catechol was almost complete when the glycine was added, and very little further O_2 uptake

occurred in the control system (curve c) which contained only catechol and enzyme; but in the presence of the secondary amines the oxidation of the glycine was particularly vigorous, and the curves of O_2 uptake were almost linear over 200 min.

DISCUSSION

The results recorded by James *et al.* (1948) showed that amino-acids containing primary amino N react with an oxidation product of catechol produced by belladonna polyphenolase to form a compound with a deep-red colour. Reasons were given for supposing the colour compound to have the structure of a *p*-amino-*o*-quinone. The results of this paper show that secondary amino N is also capable of forming strongly coloured, purple compounds. Tertiary amines, on the other hand, form no colour.

The most probable method of formation of the coloured compound is from *p*-hydroxy-*o*-benzoquinone (James *et al.* 1948) (p. 635) by condensation of the amine and the hydroxyquinone at position 4. This condensation can readily be formulated for secondary as well as primary amines, but is impossible with tertiary amines. The results of the experiments with secondary and tertiary amines may, therefore, be regarded as providing confirmation of the suggested structure for the coloured compound.

Neither secondary nor tertiary amines are themselves oxidized in the system, the uptake of O_2/mol. of catechol remaining at 2 atoms. If the secondary amine united directly with the first oxidation product, *o*-benzoquinone, to form the coloured compound, the O_2 consumption would be diminished to 1 atom. As it remains at 2 atoms, it seems safe to conclude that the quinone is first oxidized to the hydroxyquinone (consuming the second atom of O_2) as in the absence of the amine. This confirms the occurrence of the hydroxyquinone as an intermediate in the reaction chain of colour formation—and hence of amino-acid oxidation—as already suggested.

The coloured complex formed by secondary amines oxidized glycine as vigorously as an amino-acid coloured complex. The compounds derived from amino-acids, which are secondary bases, and the complexes formed from secondary amines, which are tertiary bases, are relatively stable substances in dilute solution and do not rapidly condense, like the *p*-hydroxy-*o*-quinone, to non-reducible aggregates. This relative stability is no doubt the cause of their taking part in the oxidation of amino-acids. This is clearly brought out by the results of Fig. 2 in which the addition of amino groups, after much of the *p*-hydroxy-*o*-quinone has had time to condense in their absence, brings about relatively little amino-acid oxidation.

SUMMARY

1. Secondary amines react in the belladonna polyphenolase-catechol system to produce compounds with a strong purple colour.

2. Oxygen uptake is not altered by the presence of the secondary amines.

3. Tertiary amines do not produce colours and are not oxidized.

4. The coloured complex formed by secondary amines oxidized glycine as vigorously as that formed by primary amino-acids.

5. If the addition of the amine is delayed until the catechol oxidation product has had time to condense, oxidation of glycine is reduced.

6. The above facts are considered to confirm the *p*-amino-*o*-quinone structure of the coloured compounds formed with one molecule of amino-acid. Corresponding compounds with tertiary nitrogen are presumed to result from the condensation with secondary amines. The relative stability of these compounds enables them to participate in the oxidation of additional amino-acid.

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