# THE CHEMICAL MECHANISM OF THE OXIDATIVE DEAMINATION OF AMINO ACIDS BY CATECHOL AND POLYPHENOLASE

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*Summary*

The continuous deamination of certain amino acids by the system catechol-polyphenolase takes place through intensely coloured intermediate compounds. These intermediates are formed by the combination of equimolecular proportions of *o*-quinone with amino acids or secondary amines. They are presented as structural homologues of adrenochrome or hallachrome and probably of Szent-Györgyi's tyrin (1925).

It is suggested that the continuous deamination is brought about through condensation of the colour compound with one further molecule of a suitable amino acid and rearrangement and hydrolysis into keto acid and substituted amino phenol. It is supposed that the latter is re-oxidized with liberation of ammonia and regeneration of the *o*-quinonoid colour compound. A formulation of the chemical mechanism is tentatively proposed and individual steps of the reaction are compared with observations made in the oxidation of amino acids by other polyketo compounds like alloxan and isatin. The conditions and limitations of the reaction are discussed.

## I. INTRODUCTION

Szent-Györgyi (1925) characterized a red substance "tyrin" claimed to be widely distributed in the animal and vegetable kingdom and to act as a non-enzymic hydrogen carrier in the sense of Palladin's group of respiratory pigments. The oxidative deamination of certain amino acids by the action of tyrosinase in the presence of catechol was demonstrated by Robinson and McCance (1925) and Happold and Raper (1925). The latter suggested that the reaction was due to the formation of *o*-quinone which combined with the amino acid, leading to the final decomposition of the complex with oxidation of the amino acid and formation of deeply coloured substances. Platt and Wormall (1927) showed that the formation of coloured compounds with the carrier action of tyrin could be brought about by the interaction of catechol, polyphenolase, and amino acids and "that amino-acids . . . are solely responsible for the red pigment formation with . . . the catechol-oxidase system."

The characteristics given by Szent-Györgyi for tyrin are identical with those observed for the colour compounds formed from catechol and amino acids: the compounds are intensely red to brown-red, soluble in water and alcohols, but insoluble in ether; they can be adsorbed on charcoal, but are

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difficult to elute without decomposition. They are moderately stable but prolonged standing or attempts at purification, even marked pH changes, lead to discoloration; depending on the conditions, brown suspensions or black melanin-like precipitates are formed. Reducing agents (nascent hydrogen, sulphite, certain amino acids) reduce them to leuco compounds which are readily re-oxidized by atmospheric oxygen. This redox system was further examined by James *et al.* (1948) and James and Beevers (1948) during their investigation of a polyphenolase prepared from *Atropa belladonna*. It was found that the colour compound is formed from equimolecular proportions of catechol and secondary amines or primary or secondary amino acids—but not from ammonia or primary or tertiary amines—and that no oxidation of the amino acid takes place with these proportions; but that any of these colour compounds acts as a catalyst in the oxidation of a suitable amino acid added in excess to the system.

With the belladonna enzyme no liberation of carbon dioxide took place; the ammonia liberated was quantitatively determined and was found to correspond to the excess oxygen uptake over that required for the oxidation of catechol alone (two atoms for one molecule of catechol); the keto acid could be identified in the oxidation products from glycine and alanine. The authors proposed for the colour compound the structure of a *p*-amino-*o*-quinone and related this structure to that of adrenochrome and dopa-quinone which show an identical, though weaker carrier action (Fig. 1). Jackson and Kendal (1949) investigated the purple colour compound formed when crude extracts of mushrooms oxidize catechol and identified it as consisting of equimolecular proportions of hydroxyproline and catechol according to the formula in Figure 1; their observations and conclusions confirm the results obtained by James *et al.* (1948).

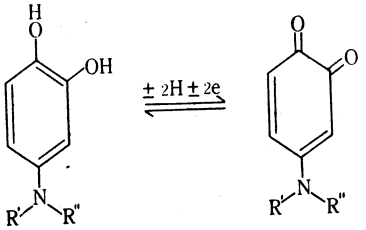



Fig. 1.—Leuco and oxy form of the colour compound.

The present work, begun at the Department of Botany, Oxford, in 1947 and continued by one of the authors (E.M.T.) at the Department of Physiology, University of Melbourne, is mainly concerned with the chemical mechanism of amino acid oxidation by the quinonoid redox system. It is intended (1) to discuss the formation and nature of the coloured compounds in more detail, (2) tentatively to present a formulation of the chemical mechanism of continuous amino acid oxidation, (3) to explain why no oxidation occurs with

equimolecular proportions of catechol and amino acids and why secondary amines or amino acids are not oxidized by the system, and (4) to consider the conditions and limitations of the reaction.

## II. Materials and Methods

The substances used were of Analar or reagent quality; no indications were found that any of them contained impurities interfering with the reactions studied. The solutions were freshly prepared before each experiment. Conditions and concentrations were chosen so as to conform with those suitable for enzymic reaction: catechol from M/500 to M/1000 and amino acids or amines, if added, in molar proportions; pH between 6 and 7.8 (at higher pH entirely different reactions occur); temperature between room temperature and 37.7°C. *o*-Quinone was prepared by the method of Willstätter and Müller (1911), adrenochrome by the method of Randall (1946), and 1,2,4-trihydroxybenzene by the method of Jackson (1939).

*Enzyme preparation.*—In the earlier part of the work the belladonna enzyme used by James *et al.* (1948) was employed; the graphs presented in the present paper were obtained by using a polyphenolase prepared from the related plant *Duboisia myoporoides* R.Br. by the same procedure: about 50 g. of *Duboisia* leaves are ground in a Waring Blendor for 3-5 minutes with 75 ml. of cold acetone, filtered, and the residue re-extracted twice with the same volume of acetone; the last extract shows only a slight green colour. The residue is then extracted three times with 30 ml. of distilled water; the last extract should give only a faint reaction for amino acids with ninhydrin and no catechol or catechin reaction with ferric chloride. The residue is again washed with acetone once and dried in a desiccator under reduced pressure; it consists of a pale whitish or brownish powder; yield: 7-8 per cent. of the weight of the fresh leaves. Kept in a desiccator the preparation is stable for several weeks; 25 mg. of it are sufficient to guarantee the oxidation of 1 ml. M/100 catechol or adrenaline without risk of inactivation. Since the enzyme preparation is insoluble in water, the reaction products can be obtained protein-free by filtration. Figure 2 shows the oxygen uptake of the catechol-polyphenolase system alone and with varying amounts of glycine in phosphate buffer, pH 7.8 at 30°C.

Some preparations gave a practically linear oxygen uptake over four to five molecules of amino acid within three to four hours of the manometric experiment; with others the rate of oxidation dropped quickly. It appeared that the *Duboisia* enzyme used in later work was more constant in its action than the belladonna enzyme with which the authors worked in Oxford.

Oxygen uptakes were measured in Warburg apparatus under the conditions mentioned; ammonia and volatile amine in the reaction products were separated by the method of Conway (1947). Colorimetric measurements were made with a Lumitron colorimeter (Photovolt, New York).

## III. Results

### (*a*) *Colour Compounds*

(i) *Formation of the Compounds under Varying Conditions.*—Catechol alone does not form coloured compounds with ammonia, amines, or amino acids under the conditions and dilutions of the enzymic reaction. On addition of oxidizing agents (ferricyanide, hypochlorite, chloramine *T*, etc.) colour formation with amino acids or secondary amines proceeds rapidly at room temperature, faster at slightly alkaline than at more acid pH; ammonia, methylamine, and tertiary amines give only slowly a brown, turbid discoloration.

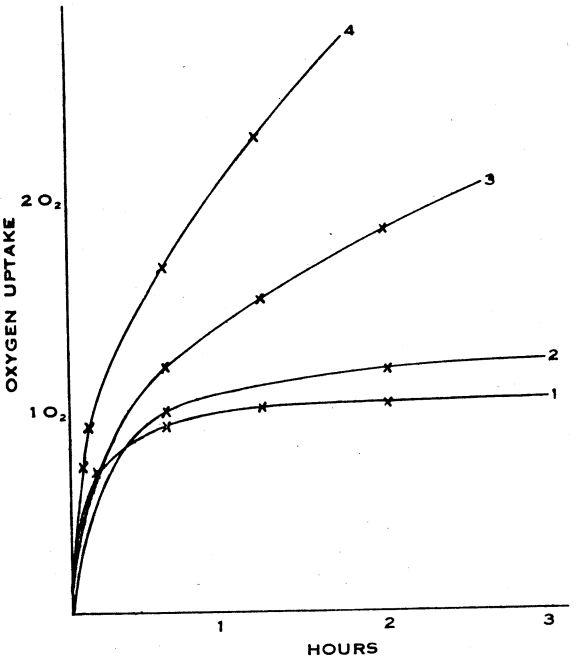



Fig. 2.—Oxygen uptake of catechol alone and with varying excess of glycine. Warburg; phosphate buffer pH 7.8; 30°C.; 100 shaking; 1 ml. M/100 catechol in final concentration of M/500. 25 mg. *Duboisia* enzyme.

Curve 1. Catechol alone.
Curve 2: Catechol plus 5 molar equivalents glycine.
Curve 3: Catechol plus 20 molar equivalents glycine.
Curve 4: Catechol plus 40 molar equivalents glycine.

The colour compounds formed are identical with those formed in the enzymic reaction and can substitute for them in the deamination of suitable amino acids (James *et al.* 1948; Jackson and Kendal 1949). With primary amino acids red-brown colours are formed. The beetroot purple formed with secondary amines and amino acids (proline, sarcosine, etc.) is distinctive and can serve as a qualitative test for the group or for the presence of a polyphenolase.

(ii) *Steps of the Reaction.*—If a dilute solution of catechol is shaken for one to two minutes with excess of the insoluble enzyme preparation and filtered, a green solution is obtained which turns quickly reddish and turbid brown. On addition of amino acid or a secondary amine to the green filtrate the characteristic colours are formed instantaneously, suggesting that the amino compounds combine non-enzymically with an oxidation product of catechol.

(iii) o-*Quinone as Intermediate.*—The addition of a green ethereal solution of *o*-quinone to water leads to a greenish aqueous solution which turns gradually reddish and turbid brown, exactly as does the filtrate from a catechol-enzyme mixture during the period of rapid oxygen uptake. If ethereal *o*-quinone is added to a dilute solution of amino acids or secondary amine, the colour compounds are formed. The solutions are as stable as those formed in the enzymic reaction.

The reaction can be further differentiated. If to an aqueous solution of an amino acid or a secondary amine, only a small amount of ethereal green *o*-quinone is added, the initial weak colour fades quickly to a brownish tint. On strong shaking with air the typical colour is formed to its full extent. With excess of *o*-quinone it is formed immediately.

The presence of the aqueous medium is, however, not essential; the colour compounds are formed equally well if ethereal *o*-quinone is added to an alcoholic solution of proline or a secondary amine or if water-free alcoholic solutions of catechol and amino compound are directly oxidized by silver oxide. Using this procedure Jackson and Kendal (1949) synthesized the compound formed from equimolecular proportions of homocatechol and hydroxyproline-ester; the compounds from amino acids were formed but could not be separated in a pure state. The present authors obtained the dimethylamine compound in clusters of small, black needles on adding ether to the reaction mixture and cooling on dry ice; the crystals decomposed, however, before the temperature could be raised to 0°C. Attempts at the syntheses are being continued. It is inadvisable to attempt the oxidation of catechol by silver oxide in the presence of undissolved or concentrated amino acids or other reducing substances; the reaction is likely to assume explosive violence.

(iv) 1,2,4-*Trihydroxybenzene as Possible Intermediate.*—*o*-Quinone in aqueous solution is supposed to be quickly hydrated to 1,2,4-trihydroxybenzene (hydroxyquinol); it was at first considered that the colour compounds may be formed by combination of the amine with the latter (James *et al.* 1948). Crystalline 1,2,4-trihydroxybenzene was, in a series of experiments, substituted for catechol in enzymic as well as in non-enzymic reactions under otherwise identical conditions. In all cases strong oxidation took place and oxidizable amino acid, if present, was oxidized, but it was not possible to obtain or demonstrate the typical colour compounds. The solutions assumed an intense dark red colour, unlike and much stronger than the colours produced from identical concentrations of catechol and amino acid. The same colour is formed in the absence of amino compounds; it is due to the semiquinone

synthesized from equimolecular proportions of 1,2,4-trihydroxybenzene and 2-hydroxy-*p*-quinone by Willstätter and Müller (1911). This colour, however, is *not* observed if catechol is oxidized by ferricyanide or by the enzyme; the solutions turn yellow or orange with a low intensity of colour and finally turbid brown. It was not possible to duplicate the result of the oxidation of catechol alone or in the presence of amino compounds by substituting 1,2,4-trihydroxybenzene for catechol.

(v) *The Mono-Substituted Colour Compound* (Fig. 1).—The observations recorded in the previous work with belladonna enzyme (James *et al.* 1948) were confirmed in the present work using a polyphenolase from the leaves of *Duboisia myoporoides* (Solanaceae).

(vi) *Effect of Excess Amino Compound on Colour Intensity.*—In the enzymic oxidation of an equimolecular mixture of catechol and a secondary amine the colour compound is formed with the uptake of two atoms of oxygen for one molecule of catechol. If further amounts of secondary amine are added to the reaction mixture, no further oxygen uptake occurs but a considerable *intensification of the colour* is observed. Its degree is difficult to ascertain in the enzymic reaction where part of the colour compound is adsorbed on the enzyme preparation. If, however, the experiment is performed by adding, to the mixture of catechol and varying molar proportions of secondary amine, ferricyanide corresponding to the oxygen uptake in the enzymic reaction—four equivalents to one of catechol—it is seen that the colour intensity rises with increasing proportions of the amino acid and reaches a distinct *maximum with the proportions of two equivalents of amine to one of catechol.* Figure 3 shows the intensity of the purple colour formed with proline after ½, 1, and 5 minutes; the blank value for catechol and ferricyanide alone, a faint yellow, is small. At room temperature the final intensity of the colour is reached within 5-10 minutes; on prolonged standing the solutions become turbid and discoloured. Hydroxyproline, sarcosine, and simple aliphatic secondary amines give similar curves.

The curves of the colour intensity seem to show a slight but constant kink at equimolecular proportions; up to this proportion they ascend steeply, almost linearly, above it they rise in a less steep, but again almost straight, line up to the proportions of two molar equivalents of secondary amine to one of catechol when they flatten out fairly abruptly.

The curves of the colour intensity formed in mixtures of catechol with varying molar proportions of primary amino acids, oxidizable or not oxidizable by the system, do not show the distinctive shape just described. The slope up to equimolecular proportions and slightly beyond is regular and there is possibly just beyond the equimolecular proportion a slight decrease in the rate at which the colour intensity increases, but there is no indication of a maximum at base : catechol proportions of 2:1. A maximum is only slowly reached with five to seven molar equivalents of amino acid (Figs. 3 and 4).

It will be noted that there is no difference between the shape of the curves formed from readily oxidizable amino acids (glycine) and those from acids which are oxidized only very slowly or not at all (glutamic acid); in view of the slow rate of amino acid oxidation no essential difference can be expected within the time of observation.

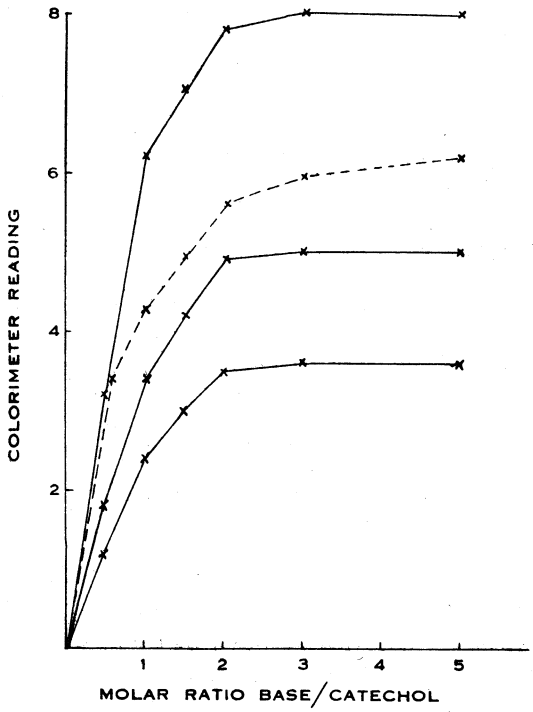



Fig. 3.—Optical density of the purple colour formed from catechol and varying proportions of proline within ½, 1, and 5 min. Catechol concentration M/1000; oxidizing agent: ferricyanide, 4 mols. to 1 of catechol, added to the mixture of catechol and proline in M/15 phosphate buffer, pH 7.8, at room temperature. The broken line indicates the optical density of the red colour formed within 5 min. from catechol and glycine under the same conditions.

(vii) *Stability of the Colour Compounds.*—Since the reaction products obtained with one and two equivalents of amine—referred to as the mono- and di-substituted colour compound—could not be separated in a pure state, it was attempted to gain further information by comparing under varying conditions the stability of the compounds formed with volatile amines. Solutions of the compounds were prepared by reacting catechol with one and two molar equivalents of dimethylamine hydrochloride and enzyme until no further oxygen was taken up (1½-2 hours) and filtering from the insoluble enzyme. Aliquots were

placed under varying conditions into the annulus of a Conway unit containing boric acid mixture (Conway 1947) in the centre well. The dimethylamine which diffused into the centre well was determined by titration with M/50

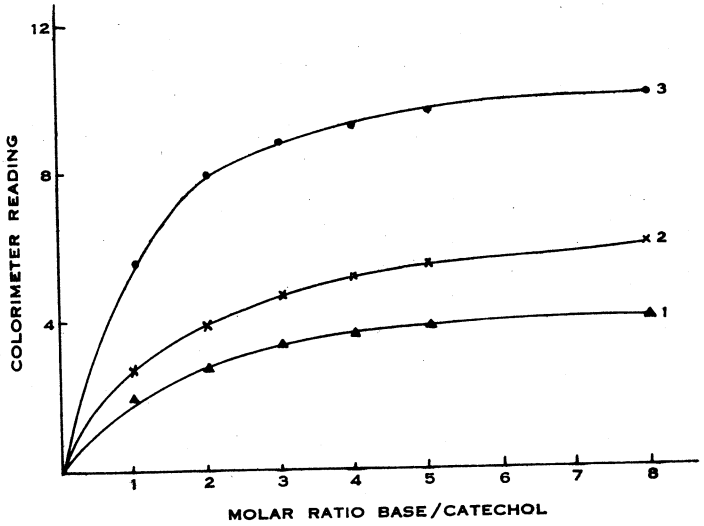



Fig. 4.—Optical density of the colour formed from catechol and glutamic acid (bottom), alanine (middle), and tyrosine (top) under the same conditions as given for Figure 3.

acid and identified by the purple colour formed with catechol and ferricyanide or enzyme. Table 1 gives the results of some experiments.

TABLE 1

VOLATILE AMINE DIFFUSED FROM THE MONO- AND DI-SUBSTITUTED DIMETHYLAMINO COMPOUNDS

| Solution Added | Colour Compound | |
|---|---|---|
| | Mono-substituted Diffused | Di-substituted Diffused |
| M/10 Phosphate buffer to pH 7.8 | 0.02 ml. M/100 | 0.03 ml. M/100 |
| M/10 Phosphate buffer to pH 8.5 | 0.022 ml. M/100 | 0.027 ml. M/100 |
| M/10 Disodium phosphate to pH 9.4 | 0.023 ml. M/100 | 0.035 ml. M/100 |
| M/100 Potassium carbonate to pH 10.5 | 0.15 ml. M/100 | 0.310 ml. M/100 |
| 0.5 ml. Conc. potassium carbonate to pH 11.4 | 0.528 ml. M/100 | 1.320 ml. M/100 |
| 1 ml. Conc. potassium carbonate to pH 11.4 | 0.642 ml. M/100 | 1.581 ml. M/100 |

Conway units containing 1 ml. M/100 colour compound in 5 ml. solution in the annulus and 2 ml. boric acid mixture in the centre well; 24 hours; room temperature. Blank values of solutions 0.015-0.030.

The results have to be interpreted with reserve, since all mono- and di-substituted colour compounds, including adrenochrome, undergo at more alkaline pH further oxidation with liberation of volatile amine. At weakly

alkaline pH and room temperature, however, the diffusion rate of dimethylamine is low and even then some oxidative decomposition cannot be excluded. The constant results are: *up to a pH of* 9 *to* 9.5 *both colour compounds are equally stable;* the amounts of dimethylamine diffused within 24 hours are within or near the blank values. Above a pH of 10 the di-substituted compound releases under identical conditions considerably higher amounts, up to one equivalent more, of dimethylamine than the mono-substituted compound, suggesting that the second molecule of dimethylamine is less firmly held than the first.

(viii) *Displacement of the Volatile Amine by Non-Volatile Amino Acid.*—If a non-oxidizable amino acid, e.g. glumatic acid, is added to either the mono- or the di-substituted compound, not much change is observed: no oxygen is taken up and very little, if any, volatile amine is released; the solutions retain their purple colour. If a readily oxidizable amino acid like glycine is added, it is oxidized and so much ammonia is formed that it is not possible convincingly to demonstrate the release of volatile secondary amine. The colour of the solution, however, changes slowly from the *purple* tint of the compounds formed with secondary amines to the *brown-red* of the compounds formed with primary amino acids. If a slowly reacting amino acid, like alanine or isoleucine is added, the colour of the solution changes likewise to a more brownish tint, but only little oxidation takes place. If the experiment is conducted in a Conway unit at a pH between 8 and 9, it is found that only very little amine diffuses into the centre well from either the mono- or di-substituted compound. If the contents of the centre well are neutralized and added to a catechol-enzyme mixture, the purple colour formed with dimethylamine is developed clearly in the liquid from the di-substituted compound, indistinctly or not at all in the contents from the mono-substituted compound. The colour change from purple to red-brown is more convincing than the diffusion of dimethylamine under conditions which exclude the alkaline decomposition of the colour compounds.

### (*b*) *Amino Acid Oxidation*

The observations recorded by James *et al.* (1948) were confirmed and need not be detailed. Figure 2 presents the results of a typical experiment.

(i) *Influence of pH.*—Nelson and Dawson (1944) state that at more acid pH the rate of formation of 1,2,4-trihydroxybenzene from *o*-quinone is markedly delayed, suggesting possibly a greater stability of *o*-quinone at the lower pH. It was found that the rate of catechol oxidation by the *Duboisia* enzyme at acid pH (5.5 to 6.5) is slightly lowered, the formation of the colour compounds, especially of those formed from primary amino acids, appears to be delayed and the rate of oxidation of an oxidizable acid is considerably slower. Experiments were conducted in which the reaction was started at acid pH and later on continued at a higher pH. Figure 5 (curves 1 and 2) shows the oxygen uptakes of catechol alone at pH 5.5 and the effect of the addition of 10 equivalents of glycine in a phosphate buffer of pH 8 after 1½ hours. Curves 3 and 4

show the oxygen uptakes of catechol plus 10 equivalents of glycine at pH 5.5 and the effect of the addition of 1 ml. phosphate buffer of pH 8 after 3 hours. It is seen that the oxygen uptakes increased immediately to about the same level as could be expected if the reaction had been started at the higher pH. Only the rate and not the course of the reaction appears to be affected by the lower pH. The big increase effected on alkalization after as much as three hours is remarkable.

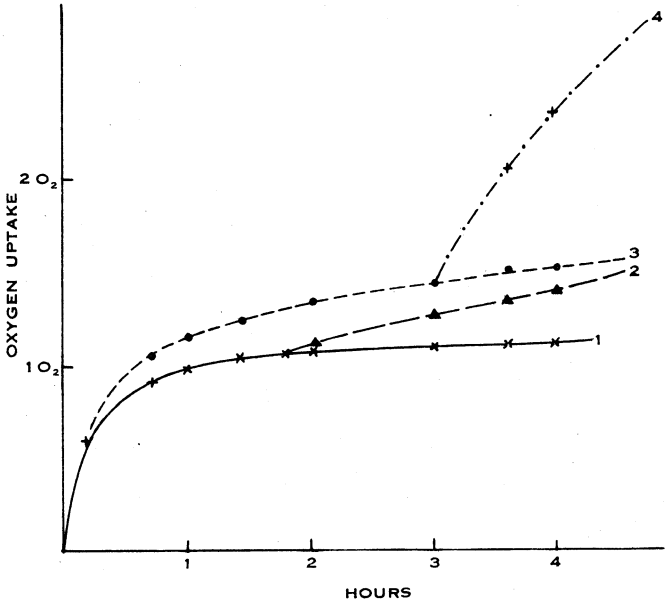



Fig. 5.—Effect of pH on the oxidation of glycine by the catechol-oxidase system. Warburg, 37°C.; 100 shaking; 30 mg. *Duboisia* enzyme; 1 ml. M/100 catechol in final concentration of M/500; initial pH 5.5; M/150 phosphate buffer.

Curve 1: Catechol alone, pH 5.5.
Curve 2: Catechol alone, pH 5.5: after 1 hr. 40 min. addition of 1 ml. M/10 glycine in M/10 phosphate buffer of pH 8.
Curve 3: Catechol plus 1 ml. M/10 glycine, pH 5.5.
Curve 4: Catechol plus 1 ml. M/10 glycine, pH 5.5: after 3 hr. addition of 1 ml. M/10 phosphate buffer of pH 8.

(ii) *Delayed Addition of an Oxidizable Amino Acid to the Catechol-Oxidase System.*—Figure 6 shows, at pH 7.8, the effect of delayed addition of 10 equivalents of glycine to the reaction mixture of catechol and enzyme at 30°C. The rate of additional oxygen uptake due to amino acid oxidation *decreased considerably on later addition* of the acid. The intensity of the colour formed decreased at the same rate as the faculty of the system to oxidize the amino acid. Addition of glycine after full oxidation of the catechol, 1½ to 2 hours, produced practically no colour and no additional oxidation. The two

functions appear to be connected. (From the graphs in Figure 5 it will be seen that the stability of the oxidizing system was greater at more acid pH.)

The solutions were examined and it was found that the *quinone reactions* (blueing of guaiacum, liberation of iodine from hydriodic acid) were strongly given by the filtrate from the insoluble enzyme during the period of intensive oxygen uptake, during the first half hour. Then they weakened; the final solutions after 1½ to 2 hours no longer gave any quinone reactions. *The faculty of oxidizing hydriodic acid disappeared parallel with that of forming the colour compounds and that of oxidizing additional amino acid.*

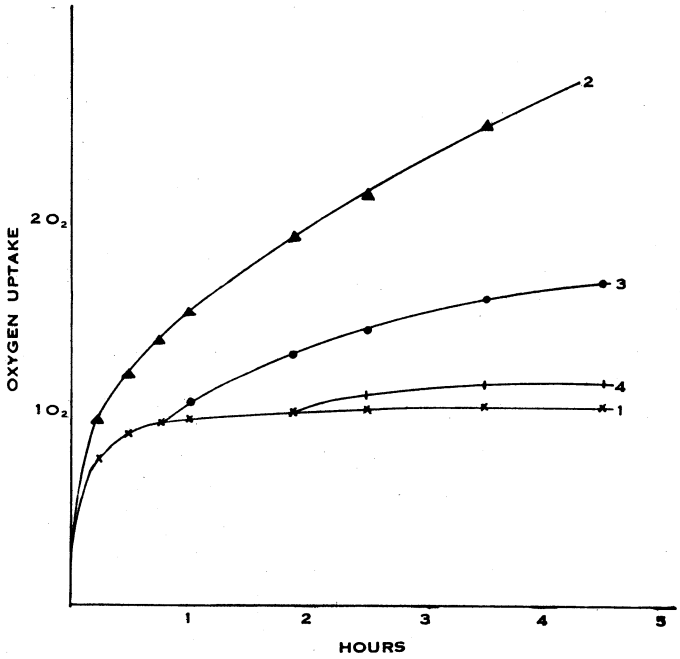



Fig. 6.—Effect of delayed addition of glycine to the catechol-oxidase system. Warburg, 30°C.; M/15 phosphate buffer pH 7.8; 100 shaking; 30 mg. *Duboisia* enzyme; 1 ml. M/100 catechol in final concentration of M/500; 1 ml. M/10 glycine.

Curve 1: Catechol alone.
Curve 2: Glycine added at the start.
Curve 3: Glycine added after 45 min.
Curve 4: Glycine added after 2 hr.

(iii) *The Reactivity of the Colour Compounds.*—In parallel experiments catechol and enzyme were allowed to react alone and in the presence of equimolecular proportions of amino acids or secondary amines to form the mono-substituted colour compounds; excess of glycine was added when the final oxidation level—two atoms of oxygen to one molecule of catechol—was reached. While the mixture containing catechol and enzyme alone showed little or no additional oxygen uptake and little or no development of colour, the flasks

containing the red compound from catechol and primary amino acids showed a considerable continuous oxidation, the rate of which was still exceeded by those containing the purple compound formed with secondary amines. (Figs. 7 and 8.) The same result was obtained when the addition of glycine was

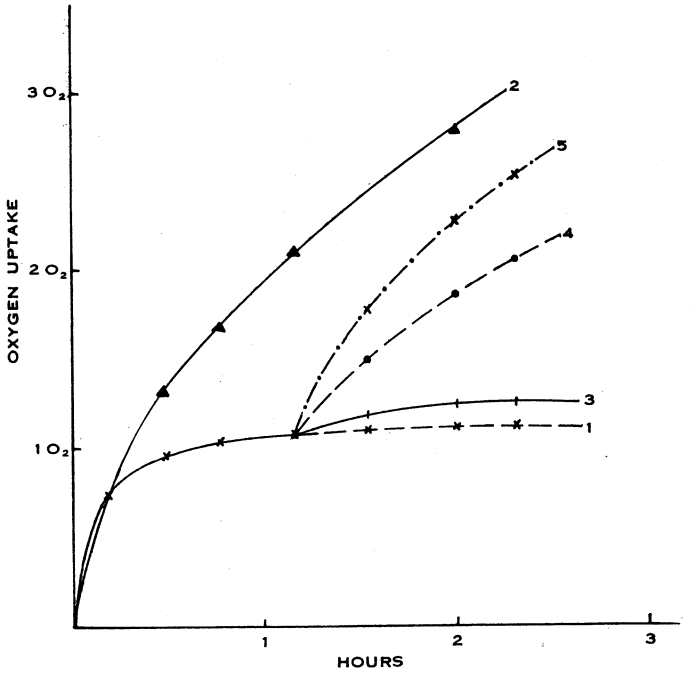



Fig. 7.—Effect of the addition of glycine to the reaction product of catechol alone and of catechol with equimolecular proportions of glycine and dimethylamine. Warburg, 37°C.; M/15 phosphate buffer pH 7.8; 100 shaking; 30 mg. *Duboisia* enzyme. 1 ml. M/100 catechol in final concentration of M/500. 1 ml. M/10 glycine was added to Expt. 2 at the start and to Expts. 3, 4, and 5 after 75 min.

Curve 1: Catechol alone.
Curve 2: Catechol alone.
Curve 3: Catechol alone.
Curve 4: 1 ml. M/100 catechol plus 1 ml. M/100 glycine.
Curve 5: 1 ml. M/100 catechol plus 1 ml. M/100 dimethylamine hydrochloride.

effected at an earlier or later stage of the experiment. *Once formed, the monosubstituted colour compounds did not lose their power of amino acid oxidation to the degree to which the catechol oxidation products lost it.*

The guaiacum reaction is unconvincing with strongly coloured solutions and even the hydriodic acid reaction presents difficulties, as the liberated iodine tends to undergo side reactions; if chloroform is overlain with a solution of potassium iodide acidified with sulphuric acid and the coloured solution is added with slight agitation, the liberation of iodine is unmistakable. It is

strongly shown by the amino acid compounds even after a few hours; the dimethylamine or proline compounds may show it after as much as 24 hours of standing at room temperature; adrenochrome shows it strongly in fresh solutions of the crystalline compound, but the reaction weakens on standing before the solutions become visibly turbid or discoloured.

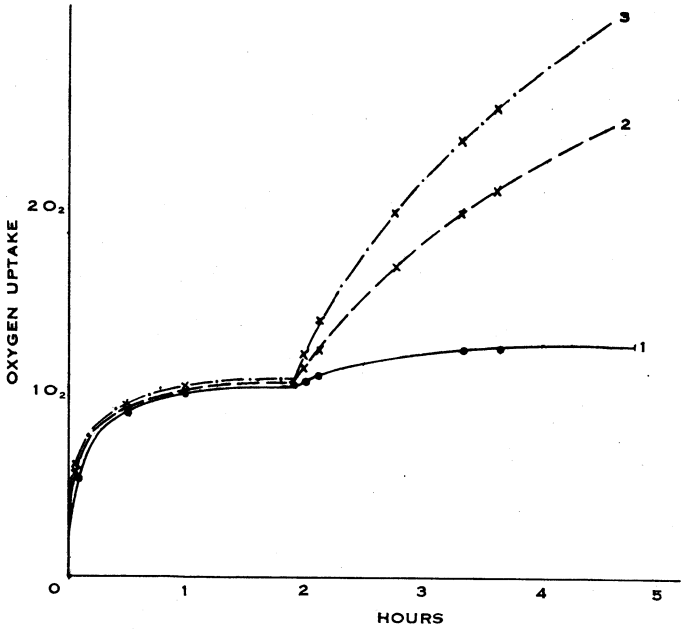



Fig. 8.—As for Figure 7 but with initial oxidation in M/100 phosphate buffer of pH 5.5. After 2 hr. 1 ml. M/10 glycine in M/15 phosphate buffer of pH 8.0 was added.

Curve 1: 1 ml. M/100 catechol.
Curve 2: 1 ml. M/100 catechol plus 1 ml. M/100 glycine.
Curve 3: 1 ml. M/100 catechol plus 2 ml. M/100 glycine.

(iv) *Effect of Secondary Amines on the Oxidation of Primary Amino Acids.*—The presence of dimethylamine or proline in moderate proportions does not lower the oxygen uptakes or oxidation rates of glycine; on the contrary it increases them. This effect is well developed already if catechol and secondary amines are present in equimolecular proportions; it is more marked if twice the amount of secondary amine is present, but still more with greater excess. There is no distinct optimal proportion of the secondary amine to catechol or to the amino acid. Figure 9 gives the result of an experiment in which a mixture of catechol with 10 equivalents of glycine was reacted alone and in the presence of 1, 2, 4, 20, and 40 equivalents of dimethylamine hydrochloride. It is seen that the rate of continuous amino acid oxidation is increased in the presence of one equivalent of secondary amine with regard to catechol and possibly slightly more in the presence of two. Still bigger proportions show no noticeable further increase and possibly a slight decrease if the excess of secondary amine becomes too big. The main difference in the oxygen uptakes occurs

*during the initial period of rapid oxidation* when the oxidizing system is being built up. Since the oxygen uptake of catechol alone is not increased by any excess of secondary amine it may be said that in the presence of the latter the oxidation of amino acid starts earlier and that a bigger or more efficient amount of the oxidizing system is formed.

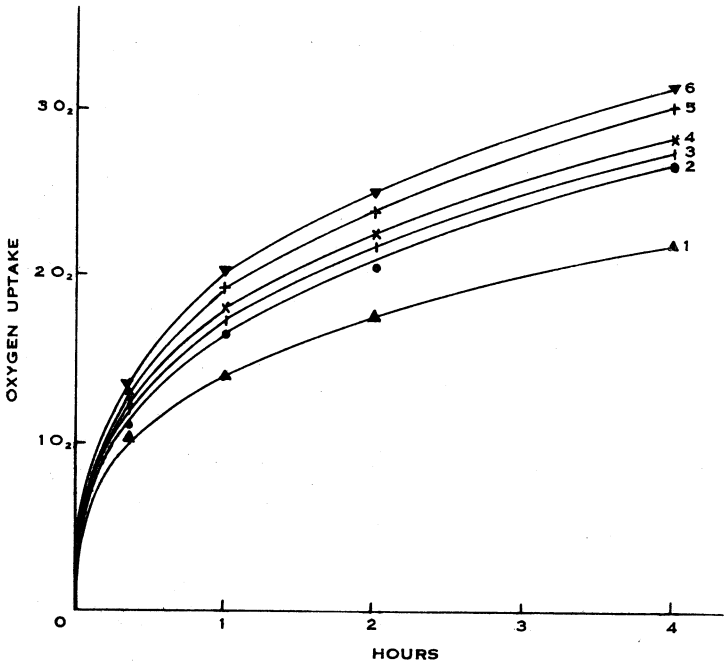



Fig. 9.—Effect of varying amounts of secondary amine on the oxidation of glycine. Warburg, 37°C.; M/15 phosphate buffer pH 7.8; 100 shaking. All flasks contained 1 ml. M/100 catechol and 1 ml. M/10 glycine. The quantities of dimethylamine hydrochloride were:

Curve 1: Nil.
Curve 2: 0.1 ml. M/10 dimethylamine hydrochloride.
Curve 3: 0.2 ml. M/10 dimethylamine hydrochloride.
Curve 4: 0.4 ml. M/10 dimethylamine hydrochloride.
Curve 5: 2.0 ml. M/10 dimethylamine hydrochloride.
Curve 6: 8.0 ml. M/10 dimethylamine hydrochloride.

## IV. Conclusions

### (*a*) *Colour Compounds*

(i) *Formation of the Mono-Substituted Colour Compounds.*—No evidence could be found that 1,2,4-trihydroxybenzene is a necessary intermediate in the formation of the colour compounds. The observations recorded are accepted as showing that the mono-substituted compounds are formed by combination of *o*-quinone with the amine or amino acid.

The reaction between quinones and aniline can proceed in two different courses (cf. Karrer 1938, p. 532): (1) *Addition* of the amino group to a double bond, followed by rearrangement of the molecule to the dihydroxyphenol and *re-oxidation at the phenolic groups with uptake of one atom of oxygen.* The original *ortho-* or *para*-quinonoid structure is thus preserved; the formulae of

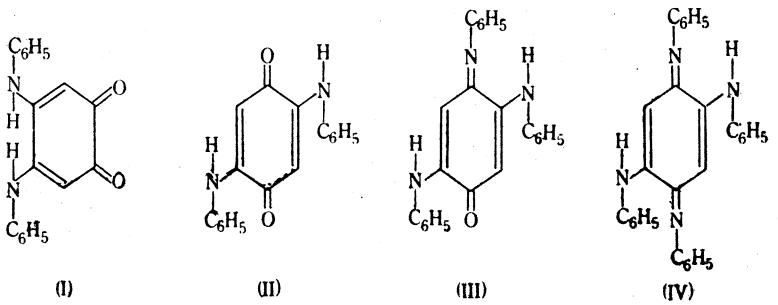



Fig. 10

4,5-dianilino-*o*-quinone (I) (Kehrmann and Cordone 1913) and of 2,5-dianilino-*p*-quinone (II) (Hofmann 1863) are given in Figure 10. (2) In the presence of excess aniline, *condensation with a keto group* of the quinone takes place, dianilino-*o*-quinonemonanil (III) (Fincke and Hagen 1885) and dianilino-*p*-quinonedianil (IV) (Kimich 1875) being formed. *No oxygen is consumed* in this reaction. All these compounds are intensely coloured. Compounds analogous to the aniline compounds have been prepared from *p*-quinone and dimethylamine (Mylius 1885) and other secondary amines; it is suggested that the corresponding compounds from *o*-quinones are the colour compounds in question.

Of the two atoms of oxygen consumed by catechol in the formation of the mono-substituted colour compounds, one is accounted for by the oxidation of catechol to *o*-quinone. The second atom of oxygen is consumed in the combination with the amine, which is therefore considered as an *addition* to one of the double bonds to form first the *leuco compound* which is then further oxidized to the *oxy compound.* It is supposed that the *ortho*-quinonoid structure is preserved exactly as it is in the reaction products of *o*-quinone with aniline. Figure 11 shows the oxidation of catechol alone (Baldwin 1947) and that of dopa and the formation of the colour compound from catechol and glycine presented as parallel reactions with identical oxygen uptakes.

No evidence was found that, in the reaction with non-oxidizable amines or amino acids, more oxygen is consumed than one atom of oxygen for one molecule of catechol. The additive reaction stops with the combination of *o*-quinone with one molecule of amino compound; the reactivity of the second double bond appears to be weakened through the first substitution.

(ii) *The Di-Substituted Colour Compounds.*—The curves of the colour intensity obtained by the oxidation of catechol with varying proportions of

secondary amine or secondary amino acid show a distinct maximum at the proportions of two of amine to one of catechol. The observation is taken as suggesting that a further molecule of the amino compound entered into the reaction. Since no further oxygen uptake occurred, it is supposed that this

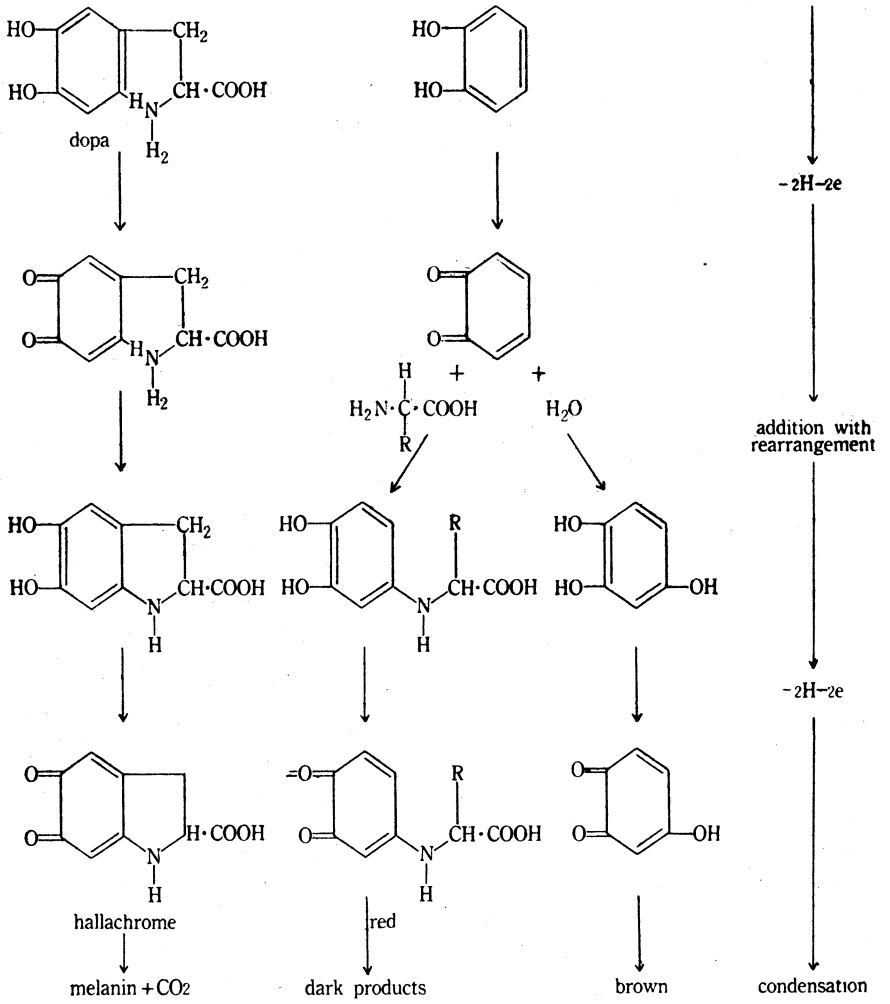
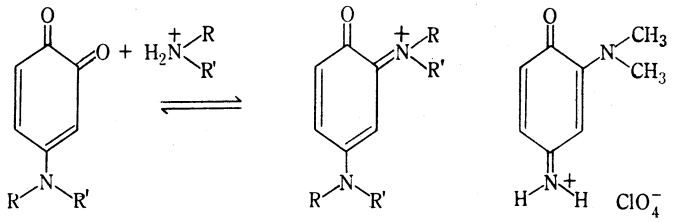



Fig. 11.—Steps of the enzymic oxidation of dopa, of equimolecular proportions of catechol and glycine, and of catechol alone.

combination is a *condensation* between one of the keto groups of the *o*-quinone and the secondary amino group, analogous to the formation of anils in the reaction between quinones and aniline. No conclusive evidence could as yet be obtained to suggest which of the keto groups is reacting. Tentatively the 2 position is proposed and accepted for the formulations of the compounds shown in Figure 12; (I) and (II); similar *p*-quinone-immonium structures are present in indamines, toluene blue, etc. Close homologues are dimethylamino-

*p*-quinone-immonium perchlorate and picrate (Piccard and Larssen 1918, Fig. 12, III). It appears that, *just as only one double bond of the o-quinone is capable of the addition reaction, so only one keto group is capable of the condensation reaction.* (The limitation need not extend to different experimental conditions.)



(I) and (II) (III)

**Fig. 12**

The curves of colour intensity appear to show a slight kink at equimolecular proportions of catechol and secondary amine, indicating possibly that the reaction proceeds stepwise: first the addition of one molecule of amine to one double bond with subsequent re-oxidation of the molecule, and then the condensation of a further molecule of the amine with a keto group of the mono-substituted compound and without further oxygen uptake.

The smooth curves of colour intensity formed in the oxidation of catechol in the presence of varying amounts of oxidizable or non-oxidizable primary amino acids do not give any indication of the formation of a condensation compound. Its formation, though possibly only to a small extent, is, however, suggested by the observations that, on addition of an oxidizable primary amino acid to the purple di-substituted compound formed with secondary amines the colour changes slowly to a more brownish tint similar to that obtained in the formation of the colour compounds from primary amino acids, and that small amounts of volatile secondary amines are released. It is supposed that the primary amino acid displaces some of the secondary amine from the di-substituted colour compound and enters the molecule at the point of displacement. Since the second molecule of a volatile secondary amine is less firmly held than the first, it is accepted that the displacement of the volatile amine by the non-volatile oxidizable amino acid takes place at the point of condensation.

It is suggested that, in the presence of sufficient amino compounds, di-substituted colour compounds are formed from amino acids as well as from secondary amines; that all these di-substituted compounds exist in equilibrium with their components and that with secondary amines the equilibrium is in favour of the di-substituted compound. The smooth curves of the intensity of the colours formed with primary amino acids are considered to be due to the facts that the equilibrium is less in favour of the di-substituted compounds and that the compounds are less stable than those from secondary amines. It is held that the formation of a di-substituted compound constitutes the first step towards the oxidation of a primary amino acid.

### (*b*) *The Mechanism of Amino Acid Oxidation*

It is suggested that the first step of amino acid oxidation consists in a rearrangement of the condensation compound from the quinone-imine to the imino acid structure (Fig. 13), analogous to the mechanism suggested for transamination by glutamic or aspartic transaminase (Snell 1945; Schlenk and Fischer 1947). It is further suggested that the quinone-imine is then hydro-

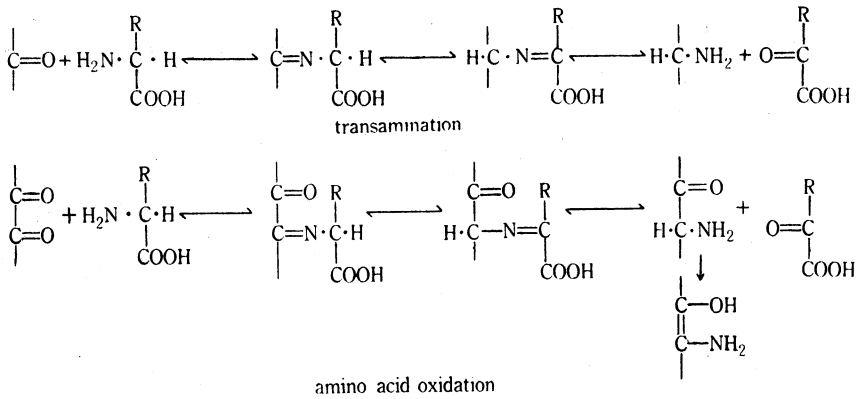



Fig. 13.—Transamination. Amino acid oxidation.

lysed into keto acid and amino phenol and the amino phenol oxidized with liberation of ammonia, thus re-forming the original mono-substituted *ortho*-quinone. The oxygen uptake for this reaction sequence, one atom of oxygen per molecule of the reduced compound, accounts for the oxygen uptake measured in the manometric experiment per molecular equivalent of oxidized amino acid. The continuous oxidative deamination of amino acids is tentatively represented by the cycle shown in Figure 14, taking catechol and glycine as examples. The same formulation can be applied to the oxidation of amino acids by adrenochrome or hallachrome, by Szent-Györgyi's tyrins, by vitamin K (Schönberg, Moubasker, and Said 1949), or other polyketo compounds, a survey of which is given by Lynen (1941).

### (*c*) *Comments on some Single Steps of the Cycle*

(i) *Nitrogen Substitution in the* 4 *Position.*—The nitrogen in the 4 position does not seem to play an active role in the subsequent reaction but to serve only in the stabilization of the *o*-quinonoid structure and in the preservation of its reactivity through repeated reductions and re-oxidations. It is considered that this effect is due less to the nature than to the fact of a substitution in the vulnerable *para* position and that carbon substitution in the same position of the naturally occurring catechins or of protocatechuic, caffeic, quinic acids etc., has the same effect. With the catechol compounds there is one further point: catechol itself is, at physiological pH in the absence of a catalyst, practically not oxidized by atmospheric oxygen, while the leuco forms of the mono-substituted colour compounds are readily re-oxidized by air. This allows a

rapid reaction in the non-enzymic oxidation and an easy investigation of the system; it may have some physiological significance. The substituted compounds may be more stable and at the same time more efficient.

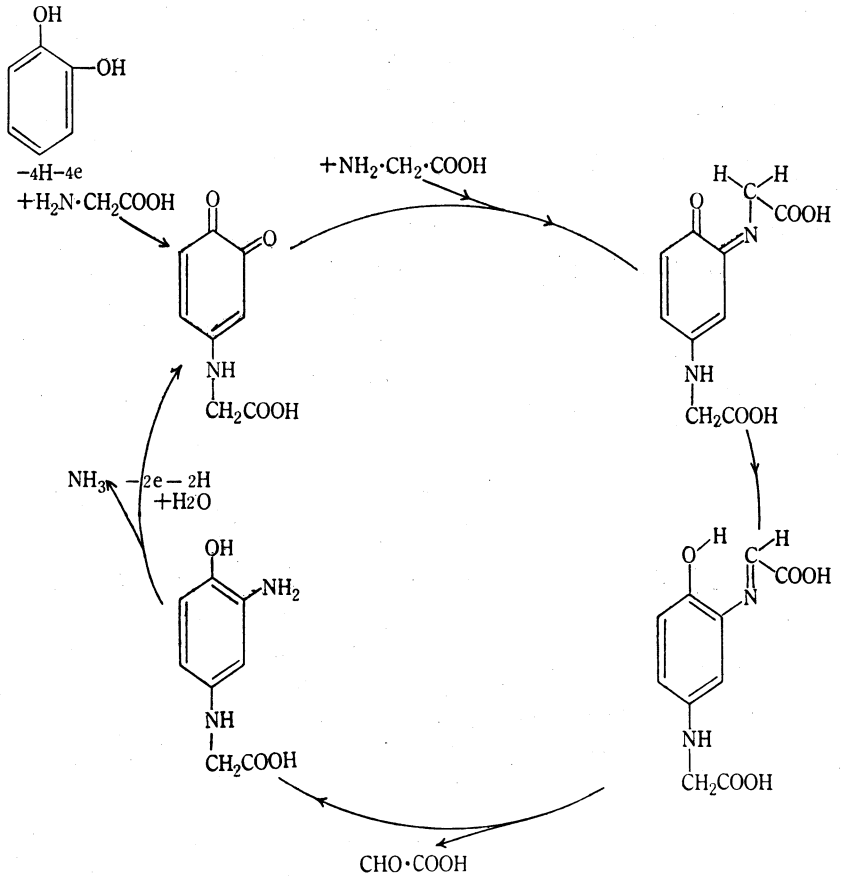



Fig. 14.—Tentative scheme of the continuous oxidation of amino acid by the mono-substituted *o*-quinonoid colour compounds.

(ii) *The Formation of the Condensation Compound.*—Neither in the slow oxidation of amino acids by the catechol system nor in that by adrenochrome could a difference in colour or any other changes be observed which would indicate the formation of the condensation compound. This step can, however, be supported by observations with another polyketo compound. Alloxan (in acetic acid) oxidizes amino acids with formation of a red intermediate; the lower aldehyde, carbon dioxide, and *some* ammonia are formed and murexide crystallizes from the reaction mixture (Traube 1911). Small amounts of uramil could be identified, but neither dialuric acid nor alloxanthin could be found. The formation of uramil proves the transfer of ammonia from the amino acid to alloxan and indicates the position at which the interaction takes place (Fig. 15). It is suggested that the red intermediate (which could not be separated

in a pure state) is the condensation compound (see Fig. 15*) corresponding to the di-substituted colour compounds formed with secondary amines and supposed for primary amino acids.

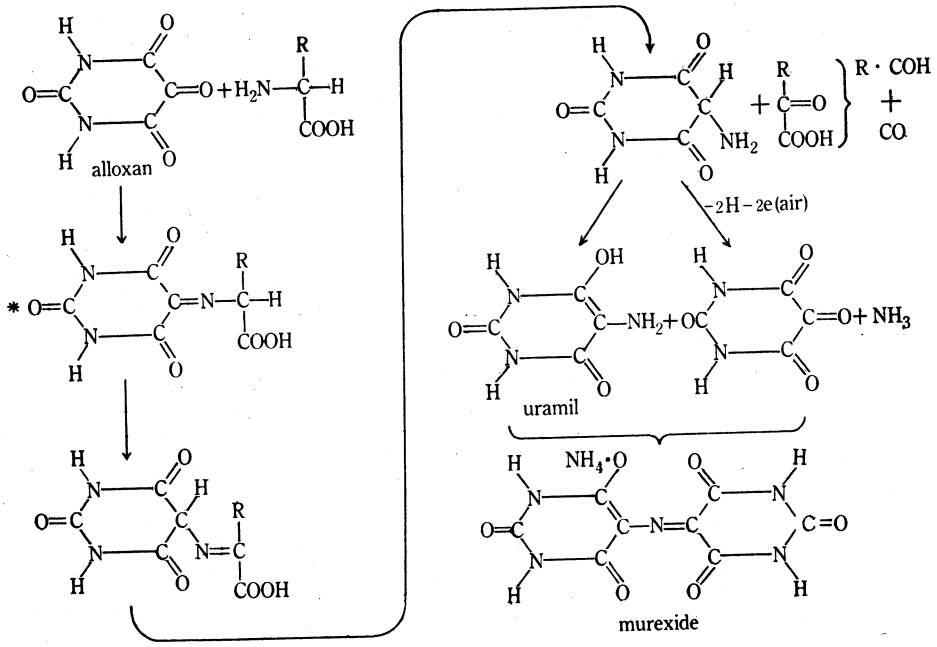



Fig. 15.—Amino acid oxidation by alloxan.

The keto acid decomposes in the boiling mixture to the next lower aldehyde and carbon dioxide; no oxygen is consumed in this step, which is a pure transamination analogous to the first step of the cycle presented. The uramil formed, however, reacts in the boiling mixture with oxygen to form ammonia and alloxan; this reaction can be presented as analogous to the last steps of the cycle. A side reaction occurs in so far as uramil, ammonia, and alloxan combine to form murexide which crystallizes from the boiling mixture and removes the catalyst. Therefore, only "some" ammonia is found in the reaction products and the process is not continuous since the oxidation of each molecule of amino acid removes one molecule of alloxan from the system in the formation of the unreactive murexide. The reaction is of interest because it presents the *formation of the keto acid—or aldehyde plus carbon dioxide—as a distinct and separate step preceding the liberation of ammonia and independent of it.*

(iii) *The Liberation of Ammonia.*—Amino phenols form on mild oxidation quinone-imines which are readily hydrolysed into quinones and ammonia. It may be assumed that in the very dilute solutions of the manometric experiment the conditions for mild oxidation are fulfilled. There are, however, some observations which suggest an alternative course for the reaction. If isatin is

is allowed to react with certain amino acids, *continuous* oxidation of the amino acid takes place and the next lower aldehyde, carbon dioxide, and ammonia are formed in almost theoretical amounts (Langenbeck 1928); isatyde is to a considerable extent present in the reaction mixture as an intermediate reduction product, which on boiling in air is re-oxidized to isatin, thus maintaining the continuous oxidation. The formation of isatyde is of interest in so far as it can be considered as the dimer of a one-step reduction product of isatin (Fig.

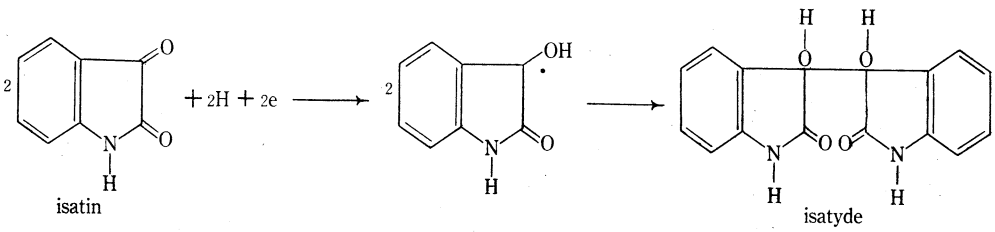



Fig. 16

16). Attempts to formulate the reaction on these lines are outside the scope of this investigation; in the present context the observation is important because isatyde corresponds to the reduced substituted catechol, the leuco compound in the cycle, indicating that *ammonia has been liberated before the re-oxidation of the reduced compound.* Pugh and Raper (1927) state that, in the non-enzymic reaction of homoquinone (3-methyl-*o*-quinone) with amino acids at neutral pH, the amino acid is oxidized and homocatechol is formed; since under the conditions the latter is not oxidized by air, the reaction is not continuous. It would appear that in this case also *ammonia is liberated before and without the oxidation of the catechol.* During the present work no evidence could be found to suggest the sequence and the mechanism of the release of ammonia.

### (*d*) *The Conditions of Oxidation*

In the cycle tentatively proposed the amine radical substituted in the 4 position of the benzene ring takes no further part in the reaction; it is neither rearranged nor oxidized to a quinone-imine structure. *Oxidation of the amino acid radical in the mono-substituted colour compounds cannot, therefore, be expected.*

With glycine, the formation of the mono-substituted colour compound is very rapid; with some other amino acids, like leucine, colour formation under otherwise identical conditions proceeds considerably more slowly and possibly to a lesser degree. This different tendency to form the addition compound may constitute a first factor to differentiate between oxidizable and non-oxidizable amino acids.

The same consideration applies to the formation of the condensation compound. It appears that only secondary amines form it quantitatively or nearly so; with primary amino acids there appears to exist an equilibrium, possibly different with different amino acids and not always favourable for the formation of the condensation compound. Even with a readily oxidizable acid like

glycine, five to ten times excess of the acid over the catechol present is needed to obtain a brisk rate of amino acid oxidation.

The rearrangement of the condensation compound to the imino acid structure is presented as a proton migration. Dimethylamine and other secondary amines do not possess an active alpha hydrogen atom as do the amino acids. *The oxidation of secondary aliphatic amines cannot, therefore, be expected,* even if they form a quinone-immonium structure.

The presence of an alpha hydrogen is not, however, sufficient to effect the rearrangement of the molecule and the subsequent deamination of the amino acid. James *et al.* (1948) found that their belladonna enzyme oxidized only glycine, ornithine, and a few other amino acids at a noticeable rate; the *Duboisia* enzyme used by the present authors shows the same limitations. Identical observations were made in the non-enzymic oxidation of amino acids by various quinones and related compounds; each system oxidizes some amino acids quickly, some sluggishly, and others not at all. The oxidizable acids are not the same in all cases, but usually the simpler acids are more readily oxidized. The varying reactivity of the alpha hydrogen almost certainly constitutes another factor determining whether a given amino acid is oxidized at an appreciable rate by the system.

The rate of continuous oxidation of a given amino acid under otherwise identical conditions appears thus to depend on the extent to which the monosubstituted compound is formed and capable of preserving its reactivity through various reductions and re-oxidations, on the extent to which the quinone-imine structure is formed, and on the reactivity of the alpha hydrogen of the amino acid.

## V. Discussion

It must be emphasized that the argument presented for the chemical mechanism of continuous amino acid oxidation by the catechol-oxidase system applies only to concentrations and conditions similar to those of the enzymic reaction. Quinones are strong oxidizing agents. Dry *o*-quinone may decompose with explosive violence in contact with the same compounds (e.g. phenylhydrazine) which in dilute solution reduce it smoothly to catechol. The observations presented on the stability of the compounds, the limitation of quinone-imine formation and the necessity of an alpha hydrogen do not apply to more concentrated solutions, to reaction at higher temperature, or to extremely slow reactions. At more alkaline pH, above 10, the colour compounds formed from catechol and amino acids as well as adrenochrome undergo further oxidation; the colour compounds from some amino acids, e.g. proline, which are not noticeably oxidized under aerobic conditions lose their colour if the access of air is restricted and regenerate it on admission of oxygen, suggesting a slow oxidation of the amino acid; α-amino*iso*butyric acid, which has no alpha hydrogen, is oxidized within eight days by *p*-quinone to acetone, carbon dioxide, and ammonia (Langenbeck 1928); and benzylamine, which has neither an alpha hydrogen nor a carboxyl group, is oxidized by isatin or alloxan

to ammonia and benzaldehyde (Traube 1911). The reaction studied in this paper takes place at room temperature and the result was considered to be negative if within 4-5 hours no noticeable oxidation was observed.

No observations were made to suggest that the colour compounds are formed through the intermediate 1,2,4-trihydroxybenzene; the evidence points to the assumption that at neutral and slightly alkaline pH in the presence of secondary amine or any amino acid the colour compound is preferentially formed and not trihydroxybenzene or any other catechol oxidation product. These colour compounds are more stable than the catechol oxidation products which rapidly lose their quinonoid reactivity.

It appears that the mechanism of enzymic oxidation is more complex than the formulation by Nelson and Dawson (1944) tends to suggest. Catechol is first oxidized to *o*-quinone and this consumes one more atom of oxygen, but the mechanism of this second step could not be duplicated by starting with the same concentrations of 1,2,4-trihydroxybenzene. Furthermore, the quinonoid reactivity is somehow lost during the second step of catechol oxidation. Willstätter and Müller (1911) succeeded in isolating the dimers of more stable substituted (3- and 4-methyl- and 4-methoxy-)*o*-quinones as yellow crystals and found that these no longer showed the quinone reactions with guaiacum and hydriodic acid given strongly by the monomers. It is tentatively suggested that *the oxidation products of catechol beyond the* o*-quinone stage present the oxidation levels of trihydroxybenzene and finally hydroxyquinone, but no longer the monomolecular structure* and that this di- or polymerization is responsible for the early loss of quinonoid reactivity, of the capability of forming the colour compounds, and of oxidizing additional amino acid.

It is suggested that, in the presence of amino acids or secondary amines, the *o*-quinone formed in the oxidation of catechol adds first one molecule of the amino compound to one of the double bonds; through rearrangement and oxidation the nitrogen substituted *o*-quinonoid colour compound is formed. All these colour compounds are presented as structural homologues; the substance tyrin described by Szent-Györgyi (1925) appears to cover individual members of this group formed from amino acids and such substituted catechols, catechins, their glucosides, or related compounds as are present in plant and possibly animal tissue.

The mechanism of continuous amino acid oxidation is presented as a condensation of the amino group of a primary amino acid with one of the keto groups of the quinone, followed by rearrangement, hydrolysis, and re-oxidation. With *o*-quinone this reaction is preceded by the addition of one molecule of amino compound to one of the double bonds followed by oxidation to a substituted *o*-quinone. No role is ascribed to this substitution in the 4 position other than that of stabilizing the compound. Alloxan, isatin, and ninhydrin do not possess double bonds which are reactive under the conditions of the experiment; therefore they do not form an addition compound, but react directly by condensation with one of the keto groups, oxidizing the first molecule of amino acid with which they combine.

In the reactions discussed the two keto groups are in the *ortho* position to each other. This is not a necessary condition. *p*-Quinone oxidizes certain amino acids, though considerably more slowly than the system described here (Langenbeck 1928). The question whether the reaction proceeds depends probably not so much on the position of the two keto groups as on their reactivity.

The discussion of the extent of the physiological function of the reaction is outside the scope of this investigation except for the following consideration: it is suggested that, in the presence of amino acids or secondary amines, *o*-quinones with free *para* positions react to form the coloured compounds described; but it is not suggested that the nitrogen substitution is necessary for the stability of the redox system, nor that catechol or its simpler homologues are the natural substrates of the system. Boswell and Whiting (1938) separated from potato tubers an unidentified catechin whose action with potato tyrosinase was identical to that of catechol or protocatechuic acid; the present authors extracted from belladonna root a catechol compound, probably a catechin, which could replace catechol in the enzymic reaction and was even superior in its action; the amounts isolated were too small to allow further identification. It can be accepted that these compounds, although forming redox systems of equal or similarly high potential, are smoother in their action and more easily controlled than the violently reactive simple *o*-quinones.

## VI. Acknowledgments

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