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The Oxidation of Catechol and Homocatechol by Tyrosinase in the Presence of Amino-acids

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In the early stages of the oxidation of catechol by crude extracts of potato or mushroom the formation of reddish purple pigment can be detected. Attention was drawn to this phenomenon first by Szent-Györgyi (1925), who achieved some degree of separation from potato juice of the substance which, together with enzyme and catechol, was responsible for the pigment formation. He suggested the name 'tyrin' for it and speculated as to its possible significance as a reversibly oxidizable hydrogen carrier. Platt & Wormall (1927), however, subsequently concluded that 'tyrin' was merely a mixture of amino-acids, and no more has been heard of it. Although, as Platt & Wormall believed, many amino-acids can cause pigment formation when present in solutions in which tyrosinase is oxidizing catechol, there is now reason to think that the amino-acid contributing most strikingly to the phenomenon originally observed is L-proline. The identification of L-proline as a constituent of mushroom extract, and the results of a study of some features of the reaction involved in the production of the pigment, are described in this paper.

METHODS

Tyrosinase preparations. These were made from the common mushroom (*Psalliota campestris*) according to the directions of Keilin & Mann (1938), but with the omission of some of the later steps in the purification described by these workers. Various preparations with Q_{O_2} values ranging between 50,000 and 500,000,* were used in the course of the work. Within these limits variation in the purity of the enzyme preparation has no qualitative effect on the reactions which are to be discussed.

Measurement of oxygen uptake. The Warburg manometric apparatus was used. The total volume of reaction mixture was 2·0 ml., the components being distributed in 0·1 M-phosphate buffer of pH 7·0. The enzyme was tipped into the substrate solution from the side arm at zero time, and in critical experiments the inset cup of the reaction vessel contained 20% KOH and a roll of filter paper. In the earliest experiments the temperature of the water bath was 20°, but was changed to 25° when the advent of warmer weather made the maintenance of the lower temperature difficult.

Colorimetry. In experiments where quantitative assessment of pigment formation was attempted a Spekker photoelectric absorptiometer was used with a 1 cm. cell and a green filter (no. 5 of Hilger set H 455, later replaced by Ilford spectrum green no. 604). The destruction of the pigment in acid solution was similarly studied, readings being taken at timed intervals after addition of acid to the pigment solution.

Measurement of pH *and hydrogen-ion liberation.* A glass electrode was used for pH measurement. When it was desired to study the hydrogen-ion liberation accompanying the oxidation of catechol in the presence of amino-acid the reaction was carried out in unbuffered solution at pH 7·0.

* Q_{O_2} values in μl./mg. dry wt./hr., substrate catechol, determined manometrically according to Keilin & Mann (1938).

A glass electrode was immersed in the reaction mixture, which was aerated with CO_2-free air or O_2. Standard NaOH was added from a microburette as necessary to maintain the pH at its initial value. The amount of alkali added was then the measure of the hydrogen ion liberated during the reaction.

RESULTS

Preliminary attempts to isolate from mushroom extract the substance responsible for the pigment formation suggested that this substance might be an amino-acid. Of the available pure amino-acids tested in the tyrosinase-catechol system, only proline and hydroxyproline gave a colour similar to that given by the mushroom concentrates. That one of these acids might be concerned was further suggested by the fact that the more active the concentrate, the lower was the ratio of amino nitrogen to total nitrogen in it. When *p*-benzoquinone was shaken with proline in ethanol a very similar colour was produced suggesting that the pigment formation in the enzyme reaction was the result of a secondary reaction of amino-acid with *o*-benzoquinone, analogous to anilinoquinone formation in the presence of aniline (Pugh & Raper, 1927).

Isolation of L-*proline from mushrooms*

The mushrooms were minced into 95% ethanol, and after standing 2–3 hr., with frequent stirring, the supernatant fluid was decanted and the tissue squeezed out in muslin in a hand press. After filtration of the combined fluids the ethanol was removed under reduced pressure and the residue taken up in water. Sufficient basic lead acetate was added to ensure maximum precipitation, and the precipitate was removed by filtration. After removal of the lead by H_2S the filtrate was taken to dryness at 30° *in vacuo* and extracted with cold methanol. The methanol was removed from the extract under reduced pressure, and the residue dissolved in water and precipitated with Reinecke salt. This precipitation was carried out in two stages, separating first the precipitate formed at 25°, and then lowering the temperature to 5°, when a second crop of Reineckate was obtained. The precipitates were decomposed separately by the method of Kapfhammer & Eck (1927), and the solutions were evaporated to dryness under reduced pressure. The material from the Reineckate precipitate obtained at 5° gave a white crystalline solid on treatment with absolute ethanol. Recrystallization from absolute ethanol gave a product of m.p. 218–220° and $[\alpha]_D^{20°}$ − 76° in water. The figures usually quoted for L-proline are m.p. 220–222° (Kossel & Dakin, 1904), and $[\alpha]_D^{20°}$ − 80° (Fischer & Zemplen, 1909). (Found: C, 52·3; H, 8·0; N, 12·0. Calc. for proline $C_5H_9O_2N$: C, 52·2; H, 7·8; N, 12·2 and for hydroxyproline $C_5H_9O_3N$: C, 45·8; H, 6·9; N, 10·7%.) It is concluded therefore that the product was L-proline. About 1 g. was obtained from 7 kg. of mushrooms.

Oxidation of catechol by tyrosinase in the presence of proline

Fig. 1 shows the course of O_2 uptake when a small amount of catechol was oxidized by excess of tyrosinase under the conditions already specified, in the presence of different molar proportions of DL-proline. No special significance is to be attached to the initial rates of uptake, which were limited by the rate of diffusion of oxygen into the liquid phase, but it is clear that in the presence of proline the uptake of the second atom of oxygen/mol. of catechol was greatly accelerated. Provided a sufficient amount of proline was present, the absorption of oxygen was completed within 10 min., and the amount then absorbed corresponds within the limits of experimental error to 2 atoms/mol. of catechol. The intense purple end product appeared to undergo no further change during the time of the experiment.

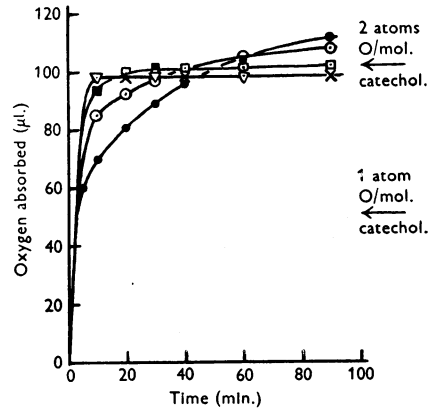


Fig. 1. Oxidation of 0·5 mg. catechol by tyrosinase in the presence of proline, at pH 7·0 and 20°. Mol. proline present/mol. catechol: ●—●, none; ⊙—⊙, 0·5; ⊡—⊡, 1·0; ▽—▽, 2·0; ×—×, 5·0.

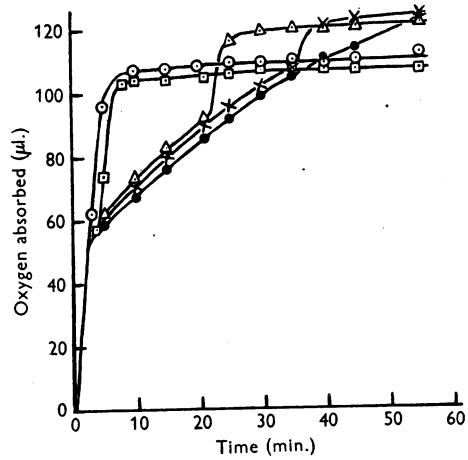


Fig. 2. Oxidation of 2·0 ml. 0·0025M-catechol by tyrosinase in the presence of 0·025M-hydroxyproline at pH 6·0 and 25°. The hydroxyproline was introduced into the reaction mixture ⊙—⊙, before the beginning of the oxidation; ⊡—⊡, at 4 min.; △—△, at 22 min.; ×—×, at 36 min.; and ●—●, not at all.

When hydroxyproline is substituted for proline in such an experiment the course of O_2 uptake and the colour developed are scarcely distinguishable from those observed in the case of proline. Fig. 2 shows the O_2 uptakes in an experiment in

which the addition of hydroxyproline to the reaction mixtures was delayed for a period after the enzymic oxidation of catechol had begun. Warburg flasks with two side arms were used, the second side arm containing the imino-acid solution which was tipped into the main part when the desired time interval had elapsed. The experiment was carried out at pH 6·0 in order to slow down the absorption of the second atom of oxygen. It will be noted that the addition of the imino acid caused in each case a rapid absorption the magnitude of which diminished with increasing delay in imino acid addition; but the total uptake at the end of the experiment was the smaller, and the nearer to precisely 2 atoms/mol. catechol, the earlier the imino acid addition. At the end of this experiment each reaction mixture was diluted to 50 ml. and its colour intensity measured in the Spekker absorptiometer. The results obtained are shown in Table 1. Little reduction in pigment formation was observed

Table 1. *Effect of delay in hydroxyproline addition on pigment formation in the catechol-tyrosinase system*

(Reaction mixture 2·0 ml., pH 6·0, 25°. 0·0025 M-catechol, 0·025 M-hydroxyproline. Diluted with water to 50 ml. for absorptiometer reading.)

Time after beginning of oxidation when hydroxyproline was added (min.)	Atoms of O/mol. catechol already absorbed at this time	Final colour intensity*
0	0	0·490
4	1·03	0·430
22	1·66	0·279
36	1·95	0·217
No addition	—	0·107

* Absorptiometer reading, filter no. 604.

when the addition of hydroxyproline was delayed until 1 atom of oxygen/mol. catechol had been absorbed, but the uptake of the second atom of oxygen was accompanied by a corresponding loss of capacity for pigment production upon addition of imino acid. Similar results were recorded by Jackson (1939) in a study of the oxidation of catechol in the presence of aniline, and were interpreted as indicating that only the primary oxidation product of catechol, i.e. *o*-benzoquinone, reacted with the base to form pigment. The same interpretation fits the facts of the present case.

Oxidation of catechol by tyrosinase in the presence of glycine

The effect of glycine on the oxidation is shown in Fig. 3. In this case, too, the initial very rapid O_2 uptake was greater in the presence of the amino-acid. At the lower glycine concentrations the absorption had almost ceased in 50 min., and the total amount absorbed at the end of the experiment was less than in the absence of amino-acid, though significantly in excess of 2 atoms/mol. catechol. At the highest glycine concentration the rapid phase was followed by a further O_2 uptake which continued at a considerable and almost constant rate until the end of the experiment. The colour developed is an orange pink, and the observations suggest that a fairly stable pigment is formed when catechol and glycine are in equimolar proportions, and that when glycine is present in excess oxidation of the excess takes place, as indicated by the continued O_2 uptake. This oxidation is accompanied by liberation of NH_3, as was observed by Platt & Wormall (1927).

A few similar experiments were made with alanine, glutamic acid and arginine, with similar results. In each case, at relatively high amino-acid concentrations, the rapid phase of O_2 uptake ending at about 2 atoms/mol. catechol was followed by a slower continued absorption indicative of oxidation of the amino-acid. The rapidity of this oxidation diminished in the series glycine, arginine, glutamic acid, alanine.

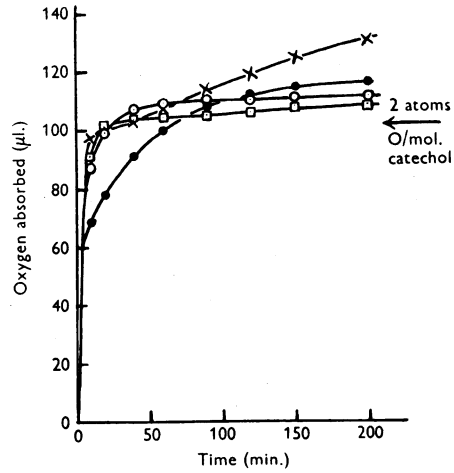


Fig. 3. Oxidation of 0·5 mg. catechol by tyrosinase in the presence of glycine, at pH 7·0 and 20°. Mol. glycine present/mol. catechol: ●—●, none; ⊙—⊙, 1·0; ⊡—⊡, 2·0; ×—×, 5·0.

Oxidation of catechol by tyrosinase in the presence of pyrrolidine

It is of interest to know whether pyrrolidine, like its derivatives proline and hydroxyproline, gives rise to pigment formation and affects the O_2 uptake in the tyrosinase-catechol system. Tests showed pyrrolidine to behave like its carboxylated derivatives. The effect of pyrrolidine on the O_2 uptake is seen in Fig. 4, where curves relating to similar experiments with hydroxyproline and with aniline are given for comparison.

Oxidation of homocatechol by tyrosinase in the presence of amino-acids

Pigment formation from homocatechol (4-methylcatechol) took place in a manner exactly analogous, so far as could be judged, to its formation from catechol. In Fig. 5 the O_2 uptakes of catechol and homocatechol under the same conditions with and without proline are compared. The reaction rate, particularly as regards the uptake of the second atom of oxygen, was slower in the case of homocatechol, but the amino-acid exerted a similar effect with each substrate. When 4:5-dimethylcatechol was present as substrate, the addition of proline had no effect on the colour which developed in the reaction mixture.

Pigment formation from monophenols and imino acids

By virtue of their monophenolase activity tyrosinase preparations are able to form pigment from phenol and *p*-cresol. The pigments formed in the presence of the imino acids appear to be identical with those formed from catechol and homocatechol respectively. The course of O_2 uptake with monophenol as substrate to the enzyme is shown in Fig. 6, and is seen to be more rapid in the presence of hydroxyproline. The total uptake is about the expected 3 atoms/mol. monophenol. The induction period before the O_2 absorption rate reaches its maximum, a well-known characteristic of the oxidation of monophenol by the enzyme, is discernible in the phenol curves, though at the high enzyme concentration used in the experiment it was very brief. It is of interest to note that the duration of this period is much diminished by the presence of the imino acid, an effect which is seen more clearly in experiments with smaller enzyme concentrations.

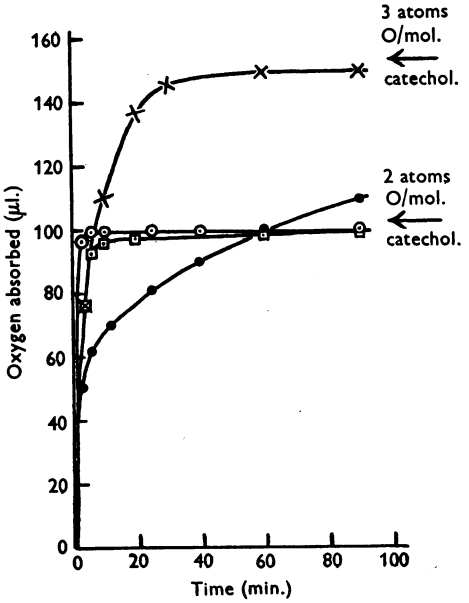


Fig. 4. Oxidation of 0·5 mg. catechol by tyrosinase at pH 7·0 and 20°, alone (●—●) and in the presence of excess hydroxyproline (⊙—⊙), pyrrolidine (⊡—⊡) and aniline (×—×).

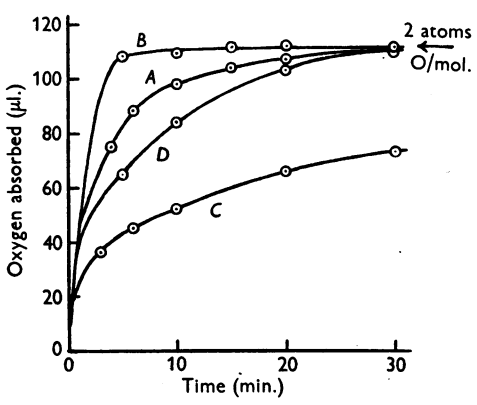


Fig. 5. Oxidation of 2·0 ml. 0·0025 M-*o*-diphenols by tyrosinase at pH 7·0 and 25°, alone and in the presence of 0·025 M-proline. *A*, catechol; *B*, catechol and proline; *C*, homocatechol; *D*, homocatechol and proline.

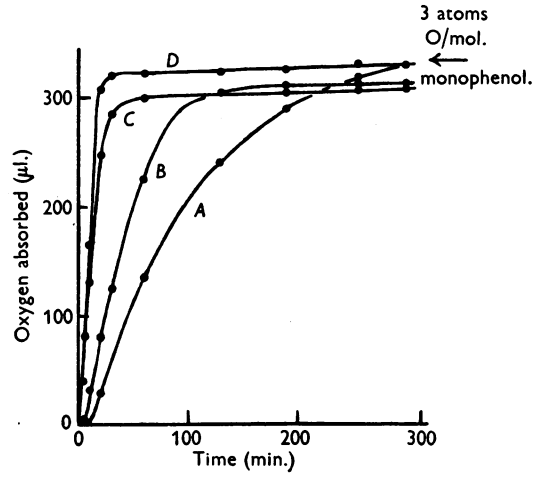


Fig. 6. Oxidation of 2·0 ml. 0·005 M-monophenol by tyrosinase at pH 7·0 and 25°, with and without 0·01 M-hydroxyproline. *A*, phenol; *B*, phenol and hydroxyproline; *C*, *p*-cresol; *D*, *p*-cresol and hydroxyproline.

In an experiment similar to that to which Fig. 6 refers, but including in addition reaction mixtures with equivalent concentrations of catechol and homocatechol instead of monophenol, the pigmented reaction mixtures at the end of the manometric experiment were equally diluted, and an estimate of relative pigment concentrations obtained by taking absorptiometer readings. The readings for the products from phenol and catechol were 0·690 and 0·678 respectively, for those from *p*-cresol and homocatechol 0·546 and 0·460. Thus pigment formation was with the former pair slightly, with the latter pair markedly, more efficient when the substrate was monophenol than when it was the corresponding *o*-diphenol.

Colorimetric study of pigment formation

To obtain information about the relationship between the extent of pigment formation and the molar ratio of imino acid to catechol during the reaction, mixtures containing 5 µmol. catechol and excess enzyme, with varying proportions of imino acid, at pH 7 and 25°, were shaken with air long enough to ensure maximum colour development, then diluted to 25 ml. with water and examined in the Spekker absorptiometer with the Ilford filter no. 604. This procedure was also carried out with homocatechol, and colour development with pyrrolidine, hydroxyproline ethyl ester, glycine, dimethylamine and methylamine, as well as with proline and hydroxyproline, was examined. The results are shown in Figs. 7 (*a*) (catechol) and 7 (*b*) (homocatechol), where the

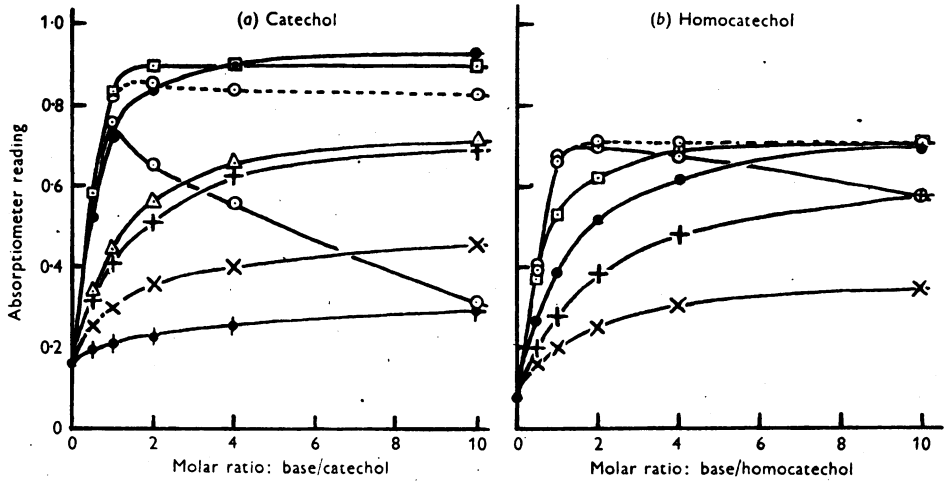


Fig. 7. Effect of variation in concentration of nitrogenous base on the colour developed during the oxidation of *o*-diphenols by tyrosinase at pH 7·0 and 25°. (*a*), catechol; (*b*), homocatechol. The broken line represents the results of an experiment in which the pH was 6·0. The bases used were ●—●, proline; ⊡—⊡, hydroxyproline; ⊙—⊙, hydroxyproline ethyl ester; +—+, pyrrolidine; ×—×, glycine; △—△, dimethylamine; ⧫—⧫, methylamine.

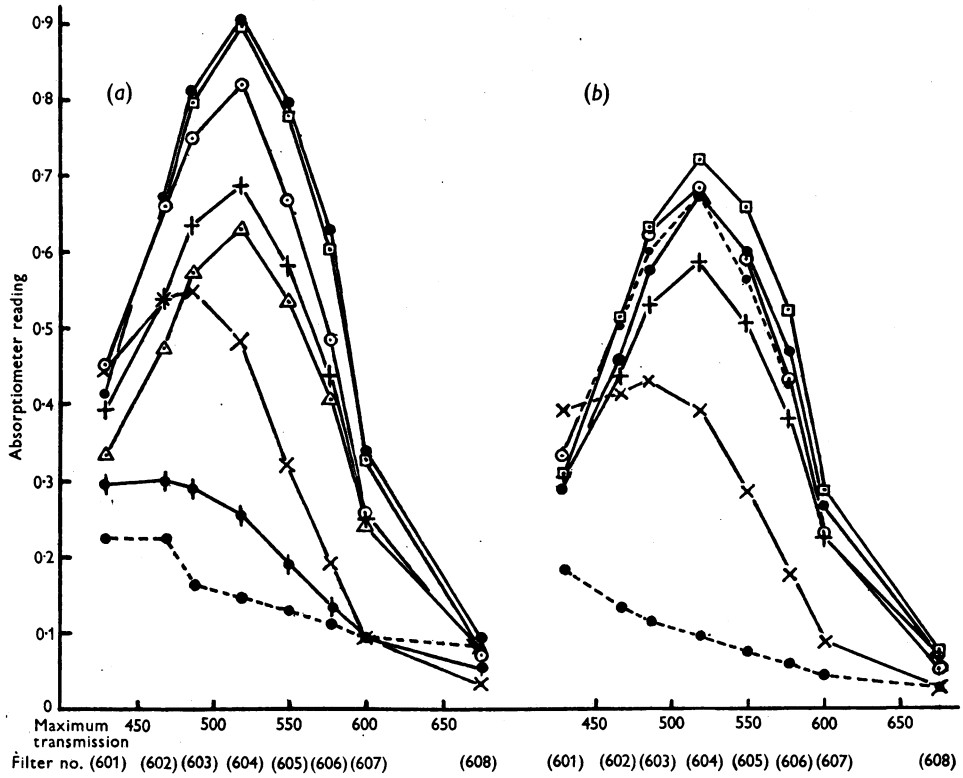


Fig. 8. Light absorption curves of pigments formed by enzymic oxidation of (*a*) catechol and (*b*) homocatechol in the presence of nitrogenous bases. Ordinates, absorptiometer readings; abscissae, wave lengths of maximum transmission of the filters used, the serial number of each filter (Ilford spectrum series) being given in the appropriate position under the wave-length scale. The bases used were ●—●, proline; ⊡—⊡, hydroxyproline; ⊙—⊙, hydroxyproline ethyl ester; +—+, pyrrolidine; ×—×, glycine; △—△, dimethylamine; ⧫—⧫, methylamine. The broken line at the foot of each set of curves indicates the readings in a control in which no base had been present. The measurements were made at a dilution corresponding to 0·0002 M in terms of the amount of *o*-diphenol used, and in a 1 cm. cell. The upper broken line in (*b*) indicates the readings given by a 0·0002 M solution of the synthetic quinone I.

colour intensity (in terms of actual absorptiometer reading) is plotted against the ratio mol. base/mol. *o*-diphenol of the reaction mixture. It will be noted that with catechol as substrate increasing the concentration of nitrogenous base above an equimolar proportion results in little increase in pigment formation in the case of proline, hydroxyproline and hydroxyproline ethyl ester, and even with homocatechol more than 50 % of the maximum colour is produced by these substances at a 1:1 ratio. This is a strong indication that the formation of the pigment requires only 1 mol. of nitrogenous base/mol. of *o*-diphenol. It seems likely that a condensation reaction takes place between 1 mol. of nitrogenous substance, through its imino group, and 1 mol. of the primary oxidation product, *o*-benzoquinone (Pugh & Raper, 1927), to give the leuco form of the pigment, which is then changed into the coloured form by the utilization of 1 atom of oxygen. In these experiments hydroxyproline ethyl ester behaved uniquely in that increasing its relative concentration above the 1:1 ratio led to a diminution in the amount of pigment found. This effect was much more striking with catechol than with homocatechol as the substrate, and was negligible at pH 6 as the appropriate curve in Fig. 7 indicates. It appears to be due to an instability of the pigment in the presence of excess hydroxyproline ethyl ester. The curves of Fig. 7 do not give such a definite indication as to the molecular proportions involved in the reactions with pyrrolidine, glycine and the methylamines. Higher concentrations of these bases were necessary for effective reaction, and it is uncertain whether an approach to quantitative conversion of *o*-diphenol to pigment was achieved even at the highest concentrations used.

The colours given by catechol and homocatechol with the same base were not distinguishable in tint to the naked eye. The various substances containing a secondary amino group appeared to give an identical colour in the reaction, whilst those with a primary amino group gave quite a different hue. To record this more objectively, absorptiometer readings were taken, using in turn each filter of the Ilford spectrum series, and the results are shown in Fig. 8. The pigments derived from secondary amines all give curves of a very similar form with maximum absorption when the green (no. 604) filter was used. The glycine-pigment curves are clearly different and show maximal absorption with the blue-green filter (no. 603), and methylamine resembled glycine in its behaviour in this respect. These facts suggest that the pigments derived from the various secondary amines are analogous in structure, but of course do not necessarily preclude a similar structure in the glycine pigment.

Liberation of hydrogen ion during pigment formation

The method used for this study has already been described. The experiments were carried out at room temperature, about 20°. The addition of enzyme to the aerated solution of *o*-diphenol and base caused a rapid liberation of hydrogen ion corresponding in time with the rapid phase of O_2 absorption and ceasing abruptly when pigment formation was completed. The course of hydrogen-ion liberation in one such experiment is shown in Fig. 9, and the relationship between total hydrogen ion liberated and amount of *o*-diphenol oxidized may be seen in the summarized data of Table 2. It is interesting to note that there was a significant liberation of hydrogen ion during the oxidation even in the absence of any nitrogenous base, but the source of this hydrogen ion is at present unknown. The data given in Table 2 are in accordance with the view that when a base was present the amine group was involved in the pigment-forming reaction, and was converted into a less basic form unionized at pH 7, so that an equivalent of hydrogen ion was liberated. Direct quantitative relation of the measured hydrogen-ion liberation to the amount of amino group reacting depends upon the assumption that the reacting group is fully ionized in its initial, and completely unionized in its final, stage. Of the bases used, only hydroxyproline ethyl ester (pK = *c.* 7·5) has its basic group appreciably unionized at pH 7, and in this case only, therefore, the experiments were carried out at pH 6. In the cases where pigment formation was most efficient, the hydrogen-ion liberation approached 1 equiv./mol. of *o*-diphenol initially present when the base/*o*-diphenol ratio was 10:1, but did not exceed this even when 90 % of the maximum was achieved at a 1:1 ratio. It is clear then that only 1 mol. of base/mol. of *o*-diphenol participates in the reaction. The

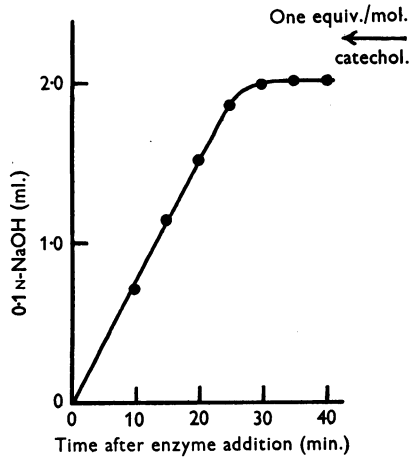


Fig. 9. Hydrogen-ion liberation during the oxidation of 0·227 mmol. catechol by tyrosinase in the presence of 0·227 mmol. hydroxyproline. The reaction took place in unbuffered solution at pH 7·0, and the ordinates represent the addition of standard alkali necessary to maintain the pH constant during the reaction.

Table 2. *Liberation of hydrogen ion during the oxidation of catechol and homocatechol by tyrosinase in the presence of nitrogenous bases*

	H^+-ions liberated/mol. of			
	Catechol		Homocatechol	
Molar ratio base/phenol	1:1	10:1	1:1	10:1
Nitrogenous base:				
Proline	0·76	0·96	0·48	0·81
Hydroxyproline	0·91	1·02	0·72	0·90
Hydroxyproline ethyl ester	0·89	0·95	0·79	0·83
Pyrrolidine	0·55	0·82	0·47	0·73
Dimethylamine	—	0·79	—	—
Glycine	0·52	0·79	—	0·68
Methylamine	—	0·53	—	—
None	0·18		0·23	

smaller yields of hydrogen ion in the case of pyrrolidine, glycine and methylamines correspond with the lesser degree of reactivity of these compounds in pigment formation already pointed out in connexion with the colour production curves of Fig. 7.

Properties of the pigments

Since the preliminary report on the formation of the proline and hydroxyproline pigments was made (Jackson & Kendal, 1940) many attempts at isolation of the pigments have been carried out without success. They are very soluble in water, methanol and ethanol, and are not extracted from aqueous solution into any of the common solvents. They are moderately stable within a narrow range of pH between 6 and 7, but even at pH 7 about 45% of the colour intensity disappears from the solution in 48 hr. at room temperature. Outside this range the pigments are very unstable, and this is undoubtedly the main difficulty in attempts at isolation.

The addition of strong acid to a solution of the pigment formed from catechol and imino acid causes rapid and irreversible decolorization, and the kinetics of this decomposition have been studied. Catechol (1 mg.) was oxidized under the conditions already described, with excess of enzyme and excess of the imino acid present. The pigment-containing reaction mixture (4 ml.) was then diluted with 0·1 M-phosphate buffer of pH 7 to a volume of 50 ml. Samples of this solution (5 ml.) were mixed with water and varying amounts of 0·1 N-HCl, to give in each case a final volume of 15 ml. Immediately after the addition of the acid the mixture was placed in the absorptiometer cell and readings were taken at timed intervals. When the desired number of observations had been made the pH of the solution was determined with the glass electrode. At pH's of less than 3·0 the pigment was found to be completely decolorized within 10 min. The results are summarized in Fig. 10, where the logarithm of the absorptiometer reading is plotted against the time which had elapsed since the pH of the pigment solution was changed from its original value of 7·0 to the value specified alongside each curve. Since the logarithmic plots are straight lines, the conversion of the pigment into colourless products has the appearance of being a monomolecular reaction. However, the relative rates of destruction at different pH's indicate that the disappearance of the colour follows the course of a bimolecular reaction in which $dx/dt = k[H^+](a-x)$, where a is the amount of pigment originally present (as measured by absorptiometer reading) and x is the amount converted to colourless products in time t. The values of k in this expression, calculated from the data of Fig. 10, are given in Table 3. The acid decomposition of the pigment formed, when catechol was oxidized in the presence of one of the partially purified fractions of mushroom extract, was examined in the same way. Log k was found to be 2·75, which is in agreement with the belief that the effective substance in the fraction was proline. In similar experiments with the pigments from homocatechol and hydroxyproline or hydroxyproline ethyl ester values for log k of approx. 3·1 and 0·4 were respectively obtained. The decolorization of the homocatechol-hydroxyproline pigment, therefore, proceeds at a rate comparable with that of the corresponding catechol pigment, whilst in the case of the ester pigment, the rate is of a different order of magnitude, some hundreds of times slower. This suggests that in the latter case a different mechanism is involved; it may be that the overall rate is determined by the rate of hydrolysis of the esterified carboxyl group present in this pigment. The pigment formed with pyrrolidine gave quite different results. Acidification of a sufficient degree produced, in this case, an instantaneous change from purple to yellow, and at no pH between 1·0 and 7·0 was any further change in the absorptiometer reading detectable in a period of 10 min. The colour at pH 1·0 was a clear yellow with no trace of pink, and intermediate tints were given as the pH was raised until the full purple colour appeared when a pH of 3·0 was exceeded. The change was reversible and the pigment behaved as a typical indicator. A rough calculation, from the absorptiometer readings, of the proportions of the two forms present at different pH's suggested that the colour change was associated with the ionization of a group with a dissociation exponent of about 2·1, whether the pigment had been prepared from catechol or from homocatechol.

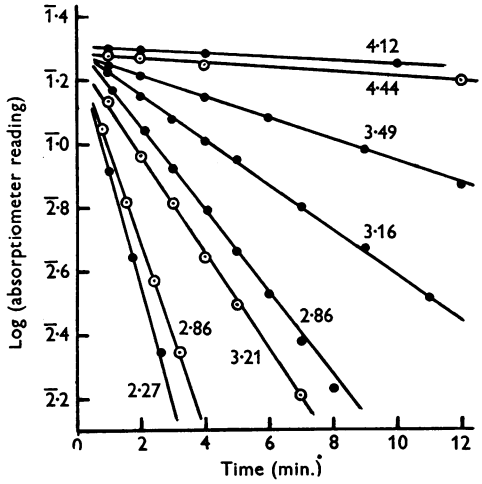


Fig. 10. Decolorization of pigments in acid solution. The logarithm of the absorptiometer reading is plotted against the time which had elapsed since the pH of the pigment solution was changed from its initial value of 7·0 to the value specified alongside each curve. The pigments were formed from ●—●, catechol + hydroxyproline; and ⊙—⊙, catechol + proline.

Table 3. *Velocity constants of the pigment decolorization reaction*

Proline pigment		Hydroxyproline pigment	
pH	log k	pH	log k
2·86	2·69	2·27	2·19
3·21	2·76	2·86	2·33
4·44	2·67	3·16	2·36
—	—	3·49	2·39
—	—	4·12	2·18
Mean	**2·71**	Mean	**2·29**

The decomposition of the proline and hydroxyproline pigments in acid solution is accompanied by the evolution of CO_2. This was demonstrated in experiments in the Warburg apparatus. O_2 uptake during pigment formation was determined in the manner already described. A second manometer with the same reaction mixture had no KOH in its inset cup, and within the vessel was a dangler containing 0·2 ml. of

2N-HCl, which was tipped into the reaction mixture after the O_2 uptake accompanying pigment formation was complete. From the resultant increase in pressure the volume of CO_2 liberated was calculated in the usual way, due allowance being made for the small initial CO_2 content of the reaction mixture which was determined in blank experiments. A few similar experiments were carried out in which pyrrolidine, glycine or alanine was used instead of the imino acid. In some of these the NH_3 content of the reaction mixture at the end of the acid treatment was determined, the reaction mixture being made alkaline, the NH_3 drawn over in an air stream into standard acid and estimated by back titration. The results of a number of such experiments are given in Table 4. It is evident from the table that the CO_2 liberation on acidification of the imino acid pigments approaches 1 mol./mol. of catechol used for pigment formation. Since this is not observed in the case of the pyrrolidine pigment (where the small figure obtained is not with certainty outside the limits of experimental error) it is probable that the CO_2 liberation is the result of instability of the carboxyl group in the pigment in acid solution. In the experiments with the glycine and alanine pigments CO_2 liberation on acidification lagged markedly behind O_2 uptake at the moment of acidification. This may be merely a reflexion of the failure of the approach to quantitative formation of pigment from catechol in the case of these amino-acids.

Table 4. *Oxygen uptake during pigment production, CO_2 formation on acidification of pigment solutions, and ammonia content of reaction mixtures after this treatment*

(Pigment was formed from 4·55 μmol. catechol in each case. In Exps. 1–4, 22·7 μmol. nitrogenous base were present, and the reaction mixture was acidified after O_2 absorption had ceased. In Exps. 5–8, 91 μmol. nitrogenous base were present, and the reaction mixtures were acidified at the times indicated. Zero time was the moment of addition of enzyme. The figures given are mol./mol. of catechol.)

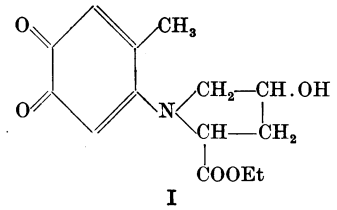
Exp. no.	Nitrogenous base	Time (min.)	O_2	CO_2	NH_3
1	Proline	—	0·99	0·92	—
2	Hydroxyproline	—	1·01	0·75	—
3	Mushroom fraction	—	1·01	0·92	—
4	Pyrrolidine	—	1·01	0·09	—
5	Glycine	17	1·10	0·20	0·61
	,,	211	3·23	0·97	2·99
6	Glycine	10	0·99	0·15	0·20
	,,	70	2·12	0·53	1·85
	,,	200	3·23	0·83	3·25
7	Alanine	10	0·88	0·64	0·00
	,,	140	1·06	0·77	0·26
	,,	220	1·21	0·83	0·51
8	Proline	210	1·01	0·95	0·00

The amounts of NH_3 liberated from the reaction mixtures were in most cases so small as to be within the limits of experimental error of estimation. Only in the later stages of the reaction with glycine can NH_3 liberation be claimed to have been with certainty demonstrated to occur on a scale too great to be accounted for as possibly the result of destruction of the pigment itself by the acid or alkali treatment. It is concluded that this NH_3 was set free in the reaction prior to the acidification; the amount of it appears to have been about 1 mol./mol. of oxygen absorbed. The probable nature of the reactions involved will be referred to in the discussion.

SYNTHESIS OF PIGMENT

In view of the difficulties met with in attempts to isolate the pigments, believed to be due to the instability of the pigments in aqueous solution, some attention was given to the possibility of preparing such pigments by ordinary chemical methods in non-aqueous media. A crystalline pigment was obtained from homocatechol and hydroxyproline ethyl ester in the following way.

Homocatechol (0·31 g.) and hydroxyproline ethyl ester hydrochloride (0·45 g.) were dissolved in dry ethanol (25 ml.) with gentle warming to facilitate solution of the hydrochloride. The addition of dried Na_2SO_4 (3·5 g.) followed by dry, alkali-free Ag_2O (1·5 g.) resulted in the development of the expected purple-red colour. The mixture was shaken for 10–15 min. Ag_2O, Ag and Na_2SO_4 were removed by filtration and the filtrate cooled in ether-solid CO_2. Scratching promoted the separation of minute rosettes of needles. After cooling (1 hr.) the crystals were filtered off and washed with light petroleum (b.p. 40–60°) until the washings were colourless, and then dried *in vacuo*. Recrystallization from absolute ethanol-ether gave 130 mg. of purplish-black glistening crystals, m.p. 125–126°. Found: C, 60·2; H, 6·3; N, 5·0. $C_{14}H_{17}O_5N$ requires C, 59·8; H, 6·4; N, 5·0%. The product is therefore taken to be 4-(*4′-hydroxy-2′-carbethoxy-1′-pyrrolidyl*)-5-*methyl*-o-*benzoquinone* with the structure I. It will be referred to in this paper as 'the quinone I'.



I

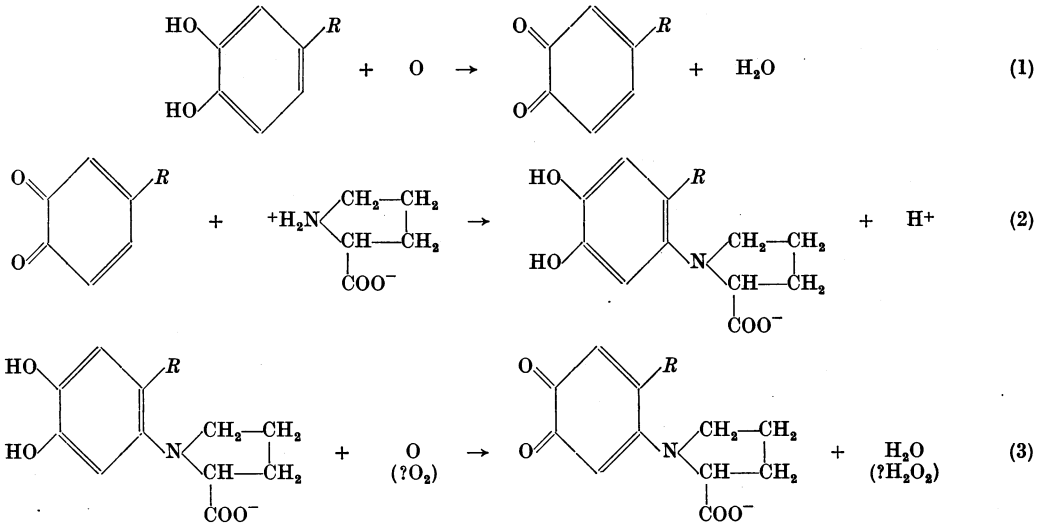
Using catechol instead of homocatechol, or free hydroxyproline or proline instead of the ethyl ester, pigments are readily formed under these conditions, but it has not so far proved possible to isolate them in a pure state. It appears that the presence in the pigment molecule of either the free carboxyl or an unsubstituted position para to the quinonoid oxygen confers upon it an extra degree of instability which renders its isolation more difficult.

Aqueous solutions of the crystalline pigment resemble very closely in colour those of the enzymically produced imino acid pigments, as may be seen on comparing the appropriate curves in Fig. 8. They are all decolorized by ascorbic acid, and in each case the leuco form of the pigment is spontaneously re-oxidized on shaking with air (e.g. after removal of enzyme by adsorption on alumina). A striking property of the imino acid pigments formed in the

enzyme reaction is that they are able to effect a non-enzymic oxidation of glycine by atmospheric oxygen, and this property is also shared by the quinone I. These facts strongly suggest that all these pigments have essentially the same structure.

DISCUSSION

The quantitative observations which have been made on oxygen uptake and hydrogen-ion liberation accompanying pigment formation, on the relationship between intensity of colour developed and imino acid concentration, and on CO_2 liberation during acid decomposition of the pigment, suggest that the sequence of reactions involved may be represented as follows:



When $R = H$ (catechol) the sequence is the same as when $R = CH_3$ (homocatechol), although the speed of the actual pigment-forming reaction (2) is greater. Of these reactions (1) is now generally accepted as the first step in the oxidation of catechol by tyrosinase. In the absence of other substances, the *o*-quinone formed takes part in further reactions which are associated with the uptake of about 1 atom of oxygen per mol. In the belief that this further uptake is precisely 1 atom/mol., Wagreich & Nelson (1938) have suggested that the product is a hydroxy-*o*-quinone which then polymerizes to give the brownish-black end product. We have frequently observed a total O_2 absorption of up to 2·3 atoms/mol. catechol, and it seems likely that the end stages are more complicated and that they may involve, perhaps in addition to hydroxyquinone formation, some degree of condensation of the type shown by Westerfield & Lowe (1942) to occur during the oxidation of *p*-cresol by peroxidase. Evidence for the accumulation of *o*-quinone during the early stages of the oxidation of catechol by tyrosinase has been provided by Dawson & Nelson (1938), and the reactions which bring about the disappearance of *o*-quinone are relatively slow compared with primary oxidation of catechol or homocatechol.

In the presence of primary or secondary amines the effectiveness of pigment formation must depend in the first place on the relative speeds of the reaction of *o*-quinone with amine, and the reactions by which the *o*-quinone is removed in the absence of amine. The condensation (reaction 2) under the conditions of our experiments appears to have been sufficiently rapid in the cases of proline, hydroxyproline, and hydroxyproline ethyl ester for pigment formation from catechol to have exceeded 90 %, from homocatechol 80 %, of the theoretical requirement according to the reaction scheme proposed. This conclusion is based on the hydrogen-ion liberation during the reactions. The reaction with pyrrolidine, glycine and the methyl amines was much slower and a greater fraction of the *o*-diphenol present was presumably converted into the same end products as are obtained in the absence of nitrogenous base.

It is well established that when catechol is oxidized by tyrosinase in the presence of aniline, dianilinoquinone is formed almost quantitatively (Pugh & Raper, 1927). It is not clear why in the case of the imino acids, reaction (2), analogous to the first stage of dianilinoquinone formation, having taken place, the quinone derived from the product by reaction (3) does not then condense with a second molecule of imino acid. No study of the products of the reaction between quinone and free aliphatic

amino-acids appears to have been made. Fischer & Schrader (1910), allowing glycine ethyl ester to react with *p*-benzoquinone in ethanol, isolated the product and showed it to be the diethyl ester of diglycinoquinone. These workers prepared in the same way the ester of dialaninoquinone, and from *p*-toluquinone the ester of diglycinotoluquinone. The formation of the *di*glycino compound from toluquinone in this case is interesting, particularly in view of Suchanek's (1914) finding that whereas *p*-benzoquinone with *p*-aminobenzoic acid gave a mixture of mono- and di-anilinoquinones, from toluquinone under the same conditions only the monoanilinoquinone was formed, while xyloquinone gave no anilinoquinone. Studies of this kind do not seem to have been made with *o*-benzoquinone, but from a reaction mixture in which *p*-cresol had been oxidized by tyrosinase in the presence of aniline Pugh & Raper (1927) isolated a dianilinoquinone-anil. The conditions under which condensations of the anilinoquinone and quinone-anil types take place do not yet appear to be very well defined. But as regards the former type of condensation the available evidence suggests that it takes place with a strong preference in a position para to a quinonoid oxygen in an *o*-quinone, and ortho to a quinonoid oxygen in *p*-quinone, without ruling out the possibility of a further condensation in a position not so situated. In the formation of the crystalline pigment prepared synthetically from homocatechol and hydroxyproline ethyl ester in the present series of experiments, the condensation was undoubtedly limited to 1 mol. of base per mol. homocatechol. There is no reason to question the identity of this pigment with the pigment formed from the same precursors enzymically and both resemble so closely the pigments formed from catechol and the secondary amines in both properties and manner of formation, that it is certain that they all have the same general structure. In the case where catechol is the substrate, it seems possible that steric hindrance by the proline residue may be responsible for the prevention of the condensation of a second molecule of imino acid in the unoccupied position para to quinonoid oxygen. The reaction of *o*-quinone with glycine appears to take place less rapidly than the corresponding reaction with imino acid, and the observations on pigment formation in the catechol-tyrosinase-glycine system do not indicate clearly the molar ratio of catechol and glycine reacting to form the pigment. It is likely that the chief reaction of glycine with *o*-quinone in aqueous solution is the same as in the case of the imino acids so that a monoglycinoquinone is formed.

A suggestion that the enzyme tyrosinase was able to bring about the oxidative deamination of amino-acids was put forward by Chodat & Schweizer (1913) and received some support from other workers. Happold & Raper (1925), however, made it clear that well dialyzed tyrosinase preparations had no effect on amino-acids other than tyrosine itself, but demonstrated liberation of ammonia from glycine and alanine by tyrosinase plus *p*-cresol, catechol or phenol. They attributed the oxidation of amino-acid to a secondary reaction of this with *o*-quinone formed by the enzyme, and showed that *o*-quinone reacted non-enzymically with amino-acids and liberated ammonia. The fact that the reaction mixtures were 'deeply pigmented' was mentioned, but without comment. The observations recorded in this paper now indicate that *o*-quinone condenses with 1 mol. of amino- or imino acid to give a leuco pigment which is readily autoxidizable in air. Whether water or hydrogen peroxide is the other end product of this autoxidation has not yet been determined. In the case of the imino acid pigments no further reaction takes place even if an excess of the imino acid is present. When the pigment is formed from glycine, and to a lesser extent when it is formed from other amino-acids, excess amino-acid is oxidized with liberation of ammonia. The relation between oxygen used and ammonia liberated in experiments of this kind with glycine (Table 4) are such as would be expected if the main end products of the oxidation of glycine were ammonia and oxalic acid. Experiments now in progress, which will be described in detail in another paper, indicate that the imino acid pigment reacts with and brings about the oxidation of glycine, and that no enzyme is required for this reaction. It therefore appears probable that the effective oxidant of excess amino-acid is a pigment with a structure corresponding to that of the synthetic quinone I. This may react with further amino-acid, dehydrogenating it to give imino acid which then decomposes with liberation of ammonia. The leuco pigment simultaneously formed is spontaneously re-oxidized by oxygen. But the possibility that an intermediate stage in the dehydrogenation is a condensation of a second molecule of amino-acid into the pigment molecule should be borne in mind.

An interesting problem arises in connexion with the decomposition of the proline and hydroxyproline pigments in acid solution. This decomposition has been found to be irreversible and to be accompanied by the loss of 1 mol. of carbon dioxide. Simple decarboxylation of the proline pigment should, however, yield the corresponding pyrrolidine pigment. However, the latter when prepared in solution directly from catechol and pyrrolidine was found to be reasonably stable, though yellow in colour, in acid solution, giving the purple form reversibly on raising the pH, and hence was clearly not identical with the decomposition product of the proline pigment. It is not at present possible to offer an adequate solution of this problem.

SUMMARY

1. The component of crude mushroom extracts which is responsible for the appearance of a purple colour when such extracts oxidize catechol has been identified by isolation of L-proline.

2. The pigment-forming reaction has been studied in respect of colour intensity developed, oxygen absorbed and hydrogen ion liberated, in systems containing purified tyrosinase, catechol or homocatechol, and one of the following: proline, hydroxyproline, hydroxyproline ethyl ester, pyrrolidine, glycine, dimethylamine and methylamine. A few experiments were also carried out with alanine, glutamic acid and arginine. The same intense purple colour was obtained in each case in which the nitrogenous substance present had a secondary amino group; the compounds with primary amino nitrogen gave a much less intense orange-red colour.

3. From homocatechol and hydroxyproline ethyl ester, by oxidation with silver oxide in ethanol, 4-(4′-hydroxy-2′-carbethoxy-1′-pyrrolidyl)-5-methyl-*o*-benzoquinone has been prepared in a pure state. It appears to be identical with the pigment formed from the same precursors in the enzymic reaction mixture, and it is concluded that all the pigments have an analogous structure, and that the pigment-forming reaction is essentially a condensation between 1 mol. of *o*-quinone and 1 mol. of nitrogenous base to give a leuco pigment which is spontaneously oxidized to the coloured form by oxygen.

4. In the case of glycine, when this is present in excess, the formation of pigment is followed by oxidation of the excess amino-acid, with liberation of ammonia. Further oxidation of this type does not take place in the case of the imino acid pigments.

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β-Glucuronidase as an Index of Growth in the Uterus and other Organs

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The β-glucuronidase activity of mouse liver or kidney has been shown to be related to the degree of cell proliferation in progress (Levvy, Kerr & Campbell, 1948). It was suggested that the rise in uterine glucuronidase observed after administration of oestrogens to ovariectomized mice (Fishman & Fishman, 1944; Fishman, 1947) could also be explained by cell proliferation.

A comparative study has been made of the kinetics of hydrolysis of phenylglucuronide by β-glucuronidase from mouse uterus, liver and kidney, and of the effects on the enzyme activities of various measures designed to produce proliferative changes in one or more of these organs. Mills (1947) showed that ox-spleen glucuronidase could be separated into two fractions, *A* and *B*, with slightly different pH optima for the hydrolysis of menthylglucuronide. Both these fractions have been found in mouse liver and kidney, while uterine glucuronidase appears to be composed entirely of *A*. No evidence has, however, been obtained to suggest that the effect of an extrinsic agent on the glucuronidase activity of an organ is dependent upon which fraction happens to be present. As in liver and kidney, changes in the enzyme level in uterus resulting from a variety of causes appear to be associated with alterations in growth.

In the course of these experiments, some unexpected changes in glucuronidase activity were encountered. In ovariectomized mice, measures