CXLVI. TYROSINASE, ITS ACTION ON PHENOLS, TYROSINE AND OTHER AMINO-ACIDS.

By ROBERT ALEXANDER McCANCE.

From the Biochemical Laboratory, Cambridge.

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Happold and Raper [1925] and Raper and Wormall [1925] produced strong evidence that tyrosinase was not capable of deaminising aliphatic amino-acids or tyrosine, and concluded that Bach's [1914] theory of this enzyme action was not correct. Robinson and McCance [1925] working simultaneously over almost the same ground, found that tyrosinase was not capable of oxidising aliphatic amino acids. Happold and Raper moreover and Robinson and McCance both confirmed Chodat and Schweitzer [1913] in this, namely that deamination of the amino-acids followed the addition of certain phenols to the system, and Happold and Raper concluded from their experiments that only those phenols capable of being oxidised to *ortho*-quinones by tyrosinase could act in this way—the amino-acid playing a purely passive part, and undergoing a secondary oxidation by the *ortho*-quinone.

The facts about resorcinol which Robinson and McCance were fortunate enough to detect, and some results reported in this paper show (1) that the system tyrosinase + phenol + amino-acid is a much more complicated one than Happold and Raper supposed, the amino-acid playing an active part in the system, and that probably no *ortho*-quinones are formed; (2) that the action of tyrosinase on tyrosine depends on the fact that tyrosine is both a phenol and an amino-acid; (3) that tyrosinase catalyses the reduction of methylene blue in the presence of an amino-acid + a phenol.

The subject will be dealt with in two parts, firstly, oxidations without enzyme, secondly, the catalysis of these by tyrosinase. All the aerobic experiments were done in Barcroft differential micro-respirometers and the anaerobic experiments in Thunberg vacuum tubes or for temperatures over 40° in test-tubes closed with a rubber bung carrying a glass tube for purposes of evacuation.

I.

Many phenolic substances show this peculiarity. They are readily oxidised by atmospheric oxygen but they are not substances which reduce methylene blue. Batelli and Stern [1921] found this with *p*-phenylenediamine, using thionine, not methylene blue. Their explanation was that the system was a

reversible one, and that with only a small amount of thionine present equilibrium was very near the start. Szent-Gyorgyi [1924] explained the same facts on the ground that the atmospheric oxygen was "activated" in Warburg's sense by iron, and on adding tissues a more rapid activation occurred, but that there was no activation of the hydrogen of the *p*-phenylenediamine by the muscle tissue, and therefore methylene blue was unable to act as the hydrogen acceptor even if tissue enzymes were present. Experiments with quinol and catechol have shown that Szent-Gyorgyi's explanation was wrong. Quinol at p_H 8 in phosphate or borate buffer was found to take up oxygen at a linear rate which was unaffected by $M/500$ concentration of cyanide (Fig. 1). This excludes activation in Warburg's sense. Further, quinol was found to reduce dinitrobenzene, where activation of the H acceptor by iron is out of

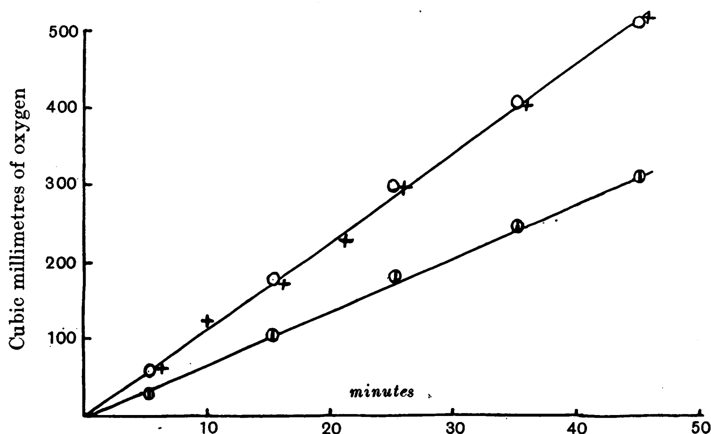

Fig. 1.

○ — ○ 22·5 mg. quinol, 3 cc. borate buffer p_H 8.
\+ — + 22·5 „ „ KCN concentration $M/500$ in 3 cc. borate buffer p_H 8.
⦶ — ⦶ 22·5 „ „ 22·5 mg. glycine, 3 cc. borate buffer p_H 8.

the question, but to do so much more slowly than it took up oxygen. The technique used was simply to incubate, after evacuation, a 1 % solution of quinol in buffer solution at p_H 8 with a little solid dinitrobenzene. A yellow colour developed over night which turned rose-pink to violet on adding soda. Quinol at p_H 8 did reduce methylene blue but only extremely slowly. The reduction was, however, complete and the rate measurable at 80°, so that there are also objections to Batelli and Stern's explanation. The experiments about to be described have made it clear that methylene blue, dinitrobenzene and atmospheric oxygen are not equivalent H acceptors. Wieland [1924] has also had occasion to note a case of this recently. Working on the amino-acid oxidation by charcoal and palladium black Wieland found that methylene blue was not capable of replacing atmospheric oxygen as hydrogen acceptor although dinitrobenzene was capable of doing so. Harger [1924] has confirmed older work that in the formation of amino-quinones from amino-compounds

and quinone by oxidation, excess of the latter can act as the oxidising agent and play the part of hydrogen acceptor with aromatic amines and amino-acid esters, but not with aliphatic amines. Atmospheric oxygen on the other hand can act as the hydrogen acceptor in all these cases. In this case quinone and atmospheric oxygen are not equivalent.

In the presence of an amino-acid, quinol and catechol both were found to reduce methylene blue (freely on warming to 80°). An example from some experimental results will make the above statement clear. From solutions of quinol (0·5 % in borate buffer at p_H 8), glycine (1 % in borate buffer at p_H 8) and methylene blue $M/1000$ tubes were filled as follows, evacuated, and placed in a water-bath at 80°.

Table I.

A. 1·5 cc. quinol + 1·5 cc. buffer + 0·3 cc. meth. blue: slightly reduced in 2½ hours
B. 1·5 cc. quinol + 1·5 cc. glycine + 0·3 cc. meth. blue: colourless in 21 mins.
C. 3 cc. buffer + 0·3 cc. meth. blue: no change in 2½ hours

This action of amino-acids was found to be specific; aliphatic amines (methylamine and ethylamine), aniline, urea and ammonia were unable to act in this way, when the p_H was carefully adjusted. Glycine anhydride had a very small effect, due no doubt to hydrolysis. This reaction has been carried out using larger quantities of the reagents to determine if the amino-acids were oxidised during the reduction of the methylene blue. 10 mg. catechol, 10 mg. glycine, 1 cc. phosphate buffer and 4 cc. $M/50$ methylene blue in phosphate buffer (p_H 8) were heated together in a vacuum, one control tube being similar but containing no methylene blue and another containing no catechol. After complete reduction of the methylene blue the amino-N content of all the tubes was estimated in the smallest form of the Van Slyke apparatus and found to be exactly the same in all. In a second experiment ammonia was estimated by Nessler's method, and no appreciable increase was detected in the experimental tube. In a third experiment catechol was replaced by quinol with similar results. It is almost certain that the oxidation quinol → quinone is not what is catalysed by the amino-acid, for if it had been the amino-acid would have been found to have been oxidised [Traube, 1911]. *p*-Cresol alone was found practically not to reduce methylene blue at all, but to do so slowly in the presence of glycine. These reactions were found to be quite unaffected by $M/500$ KCN. Tyrosine being both an amino-acid and a phenol was found to reduce methylene blue slowly. The experimental evidence is summarised in Table II. The tyrosine was recrystallised repeatedly and the rate of reduction was found to be unaffected by this treatment. 0·5 g. tyrosine was dissolved in 20 cc. phosphate buffer at p_H 8 by warming. 3 cc. (in duplicate) were added to 0·3 cc. $M/1000$ methylene blue in a tube and evacuated, placed in a water-bath at 80° and the reduction time noted. Equimolecular solutions of the other substances tested were treated in duplicate in an exactly similar way.

Table II.

Substance	Reduction time
Alanine	No reduction
Leucine	"
Tryptophan	Over 5 hours
Glycine	No reduction
Phenylalanine	"
p-Cresol	"
Tyrosine	1 hour 20 mins.

Aerobically quinol was found to have a rapid oxygen uptake alone as already stated. Addition of an amino-acid, however, did not accelerate the oxygen uptake but retarded it, and ammonia and aliphatic amines had the same effect. Both these observed results were the reverse of those obtained with methylene blue, and already described. Both seem to have their origin in the fact that there is something specific about each of these hydrogen acceptors. Fig. 1 shows the results of one out of six experiments which all gave identical results. It should be contrasted with Table I. Aerobic oxidation of catechol was found to be slower than that of quinol and the presence of ammonia to give a similar but less marked retardation of the oxygen uptake. The addition of glycine gave at first a slower oxygen uptake, as with quinol, but this oxygen uptake became faster in an autocatalytic manner. Probably further secondary oxidations follow in this case which do not occur with quinol, and the initial effect of the glycine is exactly the same in both cases. $M/500$ KCN does not affect any of these oxidations (Fig 1).

Two reactions must be capable of taking place simultaneously in the system phenol + amino-compound + hydrogen acceptor: (1) the same oxidation of the phenol as takes place in the absence of the amino-compound: (2) the oxidation of the coupled system. This alone is appreciable with methylene blue in the case of quinol, and there is a further specificity in that only amino-acids seem capable of inducing it with this hydrogen acceptor (only α-amino-acids have been tried). Aerobically both (1) and (2) take place, and amines and amino-acids exert apparently the same action. There is no unknown factor such as an enzyme present, so that all the results must be due to differences in the hydrogen acceptors. The results indicate that the various substances used as hydrogen acceptors may have certain properties in common, but that each possesses a very marked specificity of its own which is so far quite unexplained, but which must depend ultimately upon chemical structure. So much so is this the case that sometimes this chemical structure seems more important than oxidation potential. This is particularly true of the case reported and confirmed by Harger [1924] where atmospheric oxygen oxidises both aromatic and aliphatic amines with quinone to form amino-quinones, and the excess of quinone (which has an oxidation potential) will effect the former but not the latter.

The colour formed by these phenol oxidations is so striking and has formed such a large part of the evidence from which conclusions have been drawn in the past, that the following observations are important, especially if

compared with the observed rate of oxygen uptake. On adding a solution of glycine to one of catechol (both at p_H 8) there was an instantaneous violet coloration. This was due to impurities (iron, etc.) in the glycine, took place in a vacuum and, as the Barcroft results showed, had no connection with the oxygen uptake. In time the solution of catechol + glycine became darker than the solution of catechol alone, and finally went a reddish brown while the control was still practically colourless. When two bottles, one containing quinol and the other quinol + glycine, were shaken simultaneously the one containing glycine quickly became pink, then red, and later red brown. The cause of this was almost certainly the formation of amino-quinones which are red compounds thus: quinol $\rightarrow$ quinone + 2 amino-acid $\rightarrow$ amino-acid quinone.

II.

Two sources of tyrosinase have been used: (*a*) the same enzyme and method of preparation used in a previous paper [Robinson and McCance, 1925], (*b*) tyrosinase from the meal-worm. This was freshly prepared for each experiment, as the preparation soon lost its activity on keeping. The worms were ground up very fine in a mortar with a little water, the solid material washed six times with water, and separated from the washings each time by the use of a centrifuge. The washings were disregarded and the fine suspension used as the enzyme solution. The action of each enzyme appeared to be the same. The suspension of meal-worm used as a source of tyrosinase contained a powerful peroxidase.

The reduction of methylene blue in each of the following systems was found to be catalysed by tyrosinase: quinol + glycine, catechol + glycine, and especially *p*-cresol + glycine (all in buffer at p_H 8). Without enzyme quinol (or catechol) + glycine reduced methylene blue much faster than *p*-cresol + glycine but in the presence of the enzyme these relative rates were reversed and the same amount of methylene blue was reduced very much faster by *p*-cresol + glycine. *p*-Cresol is of course much more akin structurally to tyrosine than is quinol. *o*-Cresol + glycine practically did not reduce any methylene blue with or without enzyme, and *m*-cresol + glycine reduced methylene blue almost as fast as *p*-cresol in the presence of the enzyme. Tyrosinase can act therefore by accelerating the anaerobic oxidation of a phenol in the presence of an amino-acid and it is reasonable to suppose from what is known of other enzymes that this is the method by which it acts aerobically. In other words tyrosinase exerts its catalytic action by hydrogen activation. At the same time tyrosine + tyrosinase did not reduce methylene blue any faster than tyrosine alone. These experiments have been done most carefully: some tubes were kept 8 days in a high vacuum with only small amounts of methylene blue present, and Raper and Wormall's [1923] results confirmed. There can, however, be little doubt that in the presence of a suitable hydrogen acceptor tyrosinase would act on tyrosine anaerobically.

Aerobic experiments with tyrosinase afforded further evidence that the amino-acid plays an active part in the system. The oxidation of *p*-cresol is enormously accelerated by tyrosinase (its rate of oxidation without any enzyme is negligible). The rate of oxygen uptake was, however, still further increased by the addition of glycine.

Nature of the effects of amino-compounds on phenol oxidation.

Quinol has been found to form various addition compounds with aliphatic amines [Harger, 1924] and it is probable that compounds of a similar nature are formed by amino-acids and ammonia but they have not yet been isolated. It is possible that these compounds form the first step in the formation of amino-quinones. Such an addition compound might be more easily attacked by oxidising agents than the simple quinol, and in fact have quite different properties. No other phenols have so far been reported to form these addition compounds, but catechol and resorcinol have now been found to form well defined crystalline compounds of this type (see experimental section at the end of this paper), and when solutions of *p*-cresol and various amines were mixed there was an evolution of heat but no crystals were obtained on evaporation. The same was true of leucine ethyl ester with all the phenols tried, namely quinol, *p*-cresol, *m*-cresol and catechol. With Mr Dixon's assistance the effect on the reduction potential of adding glycine to a solution of catechol or quinol was tested and found to be nil. This was unexpected in view of the marked effect the same addition would have had on the reduction of methylene blue and the rate of oxygen uptake, besides the chemical evidence of a phenol-amine addition compound. The exact nature of the effect exerted by an amino-compound on the oxidation of a phenol, and the chemistry of that oxidation must remain undecided for the present.

The effect of cyanide and pyrophosphates.

Only the meal-worm tyrosinase has been used for these experiments. Both KCN and pyrophosphates have no effect on the enzyme-free oxidations already described. Pyrophosphates ($M/500$) were found to inhibit slightly both the methylene blue reduction and oxygen uptake of *p*-cresol + glycine + tyrosinase. KCN is extremely toxic to the enzyme, and exerts this effect both with methylene blue and oxygen. The following is a typical experiment. Four vacuum tubes were made up as follows: *A* and *B*; 10 mg. *p*-cresol, 10 mg. glycine, 1·5 cc. borate buffer p_H 8, 0·3 cc. methylene blue $M/1000$, 1 cc. enzyme, 0·5 cc. H_2O: *C* and *D*; the same with the addition of KCN (titrated to p_H 8) instead of water to make a final concentration of $M/500$. After evacuating, all four were placed in a water bath at 38°. *A* and *B* were completely decolorised in $1\frac{3}{4}$ and 2 hours respectively, *C* and *D* were still blue after 5 hours. There was, however, aerobically a very unusual feature of this inhibition by KCN, for its effect wore off after a time and the system began

to take up oxygen, and this moreover very much sooner and faster when glycine was present. This was definitely not due to hydrolysis of the KCN as time went on, and in fact the addition of more KCN when the latent period was over seemed to make very little difference. The enzyme seemed almost to have become immune to KCN (Fig. 2). This inhibition is obviously a very complicated mechanism and would have provided material for a complete investigation. One difficulty would have been to standardise the conditions, for the enzyme preparation seemed to lose strength on keeping, and the length of the latent period was found to depend among other things

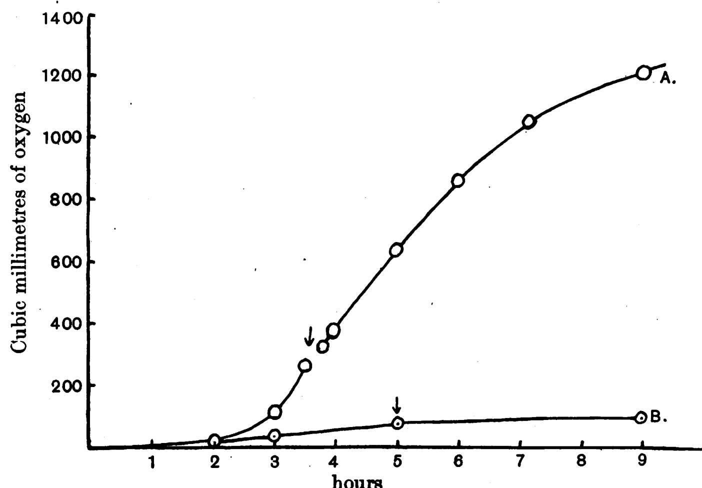

Fig. 2.

A. 10 mg. *p*-cresol. 10 mg. glycine. 1·5 cc. borate buffer p_H 8. KCN (total concentration *M*/500). 0·5 cc. H_2O. 1 cc. tyrosinase suspension.

B. 10 mg. *p*-cresol. 1·5 cc. borate buffer p_H 8. (KCN concentration *M*/500.) 0·5 cc. H_2O. 1 cc. tyrosinase suspension.

At ↓ concentration of KCN was increased to *M*/250.

on the relative proportions of the KCN and enzyme. Some of the results obtained are here given briefly: (*a*) the KCN inhibition of the oxygen uptake of *p*-cresol + tyrosinase was greatly shortened by the addition of glycine to the system; (*b*) the rate of oxygen uptake and the amount of oxygen taken up after the latent period depended on the amount of glycine present; (*c*) the red colour formed by this oxidation in the solution and, therefore, probably the products of the oxidation, appeared identical with those formed in the absence of KCN.

Perhaps the most interesting points at present are, (*a*) that there is a marked inhibition by KCN which is not an effect of iron, (*b*) that this latent period and curve of oxygen uptake is exactly the same as that obtained with resorcinol and glycine, resorcinol [Gortner, 1911] being known to exert an inhibitory effect on tyrosinase. It almost seems as if the amino-acid exerted a double function, (*a*) an effect on the phenol, (*b*) a protective effect on the enzyme.

The nature of tyrosinase.

Onslow and Robinson [1925] have advanced a theory of the action of tyrosinase, which is an extension of Onslow's "catechol" theory of oxidations. Essentially the theory is that either all samples of tyrosine contain traces of an *ortho*-dihydroxy-compound as an impurity, or that the enzyme contains traces of active oxygen. If this were true, the initial rate of oxygen uptake would depend entirely on the amount of this impurity and the curve of oxygen uptake would be autocatalytic, which has never been proved. Moreover, the following experimental evidence makes it practically certain that the oxidation of *p*-cresol, tyrosine, resorcinol, quinol, etc., with or without enzyme, and with or without glycine, or other amino-compound, does not lead to the formation of *o*-dihydroxy substances. Catechol and all *o*-dihydroxy substances combine quantitatively with boric acid to give compounds with strongly acid properties [Lambert, 1889]. This property of borates is so definite that it has formed the basis of two methods for the volumetric estimation of boric acid. All the above phenols undergoing oxidation spontaneously or under the influence of tyrosinase have been found to give quantitatively similar oxygen uptakes in borate or phosphate buffer at p_H 8 and the p_H after the oxidation is the same in both cases, *e.g.* in both 1 mol. *p*-cresol takes up 3 atoms of oxygen. On the contrary, catechol, 0·5 % in borate buffer p_H 8, gives a strongly acid solution. These same facts also disprove Happold and Raper's [1925] theory that deamination of the amino-acid follows only if an *ortho*-quinone is formed in the oxidation of the phenol.

Raper and Wormall [1925] found that tyrosinase would not act upon *p*-hydroxyphenylpyruvic acid + NH_3, though of course it would act readily on *p*-hydroxyphenylalanine (tyrosine). Methylene blue has been found to be reduced by a phenol + an amino-acid but not by a phenol + NH_3, and this reduction is catalysed by tyrosinase. It would seem that in both these cases the presence of the amino-acid was necessary to effect the oxidation of the phenol. In tyrosine both the oxidisable phenol group and the "co-enzyme" amino-group are combined in the same molecule. This seems to be the reaction catalysed by tyrosinase, though the chemistry of this oxidation remains to be determined.

Robinson and McCance [1925] reported that in the systems catechol (or *p*-cresol) + amino-acid + tyrosinase, the oxygen used for the amino-acid agreed quantitatively with the reduction of the amino-N but much less ammonia than this was set free. The explanation of this is possibly as follows. Some of the amino-acid is involved in the formation of the phenol-amino-compound (such as an amino-quinone); this would lead to a decrease in the free amino-N of the system but not to any liberation of free ammonia. Hydrogen peroxide probably is simultaneously formed and this would react with the peroxidase and more amino-acid to give both a reduction of amino-N and an increase of free ammonia. The discrepancy does not seem to be due to

the formation of cyanates, for none were detected by the methods described by Fearon and Montgomery [1924] in a solution of *p*-cresol, glycine and tyrosinase which had been aerated $2\frac{1}{2}$ days at p_H 8.

EXPERIMENTAL SECTION.

Preparation of the addition compound (catechol)$_2$-diethylamine.

2·5 g. catechol were dissolved in 50 cc. absolute ether and 5 cc. diethylamine added. Crystals very soon began to separate. These were filtered off, washed twice with small quantities of absolute ether and recrystallised from warm benzene. The crystals were white rectangular plates, very soluble in water and ether, less soluble in benzene and light petroleum. M.P. 78–79°.

Analysis. Found: C, 66·03; H, 7·93; N, (1) 4·6, (2) 4·8 %.

Calc. for (catechol)$_2$-diethylamine: C, 65·53; H, 7·86; N, 4·78 %.

After keeping a month the crystals had turned brown. The compound was completely dissociated in acid solution and the amine could be titrated.

Preparation of the addition compound (resorcinol)$_2$-diethylamine.

1 g. resorcinol was dissolved in absolute ether and diethylamine added drop by drop until the granular precipitate which appeared momentarily with each drop persisted. On standing a heavy precipitate appeared. This proved to be readily soluble in excess of diethylamine. The precipitate was filtered off, washed and recrystallised from benzene. The crystals obtained were white needles with similar solubility to the catechol compound. M.P. 115°.

Analysis. Found: C, 65·55; H, 7·71; N, 4·75 %.

Calc. for (resorcinol)$_2$-diethylamine: C, 65·53; H, 7·86; N, 4·78 %.

The crystals did not turn brown on keeping, even after 6 months.

In an endeavour to discover the nature of the compound formed by the action of tyrosinase on *p*-cresol alone, the addition compound of quinone-(*p*-cresol)$_2$ analogous to the known pheno-quinone compound was synthesised. This compound has an orange colour identical in appearance with that formed by the *p*-cresol tyrosinase system, but the properties of the two solutions are not the same.

1·5 g. quinone dissolved in hot light petroleum were added to 6 g. of *p*-cresol also dissolved in hot light petroleum. On cooling a heavy crop of orange-red crystals was obtained and recrystallised from the same solvent. M.P. 63·5°. The crystals were needles and always had a faint smell of quinone.

Analysis. Found: C, 73·78; H, 6·34 %.

Calc. for quinone-(*p*-cresol)$_2$: C, 74·1; H, 6·18 %.

SUMMARY.

1. Experiments have shown that there may be a marked specificity among the various substances classed as H acceptors. Their chemical structure seems to be the basis of this and any further explanation seems impossible at present.

2. Tyrosinase catalyses the reduction of methylene blue by a solution at p_H 8 of *p*-cresol + glycine. Tyrosinase, therefore, acts by hydrogen activation.

3. The action of tyrosinase on tyrosine depends upon the fact that tyrosine is both an amino-acid and a phenol, and though the amino-acid does not undergo any deamination, it appears to act as a co-enzyme for the oxidation of the phenol. Similarly, in the system *p*-cresol + amino-acid + tyrosinase, the amino-acid does not merely undergo secondary oxidation but plays an active part in the system.

4. The spontaneous oxidation of all the phenols examined (alone or together with amino-compounds) has been shown to be independent of KCN. *M*/500 KCN strongly inhibits both the aerobic and anaerobic action of tyrosinase.

5. Addition compounds of catechol and resorcinol with diethylamine, and a *p*-creso-quinone compound analogous to the known pheno-quinone, have been prepared for the first time.

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