CXXI. TYRAMINE OXIDASE.

I. A NEW ENZYME SYSTEM IN LIVER.

By MARY LILIAS CHRISTIAN HARE (*Bathurst Student, Newnham College*).

From the Biochemical Laboratory, Cambridge.

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The properties of tyramine, *p*-hydroxyphenylethylamine, have hitherto been studied almost entirely from the physiological, rather than from the biochemical standpoint. Dale and Dixon [1909] have reported that its effects on injection are similar to those produced by adrenaline, though less well marked. It is known to be one of the products produced from protein by the action of certain bacteria in the intestine of the higher animals, and toxic conditions occur simultaneously with increased absorption of these products into the blood stream. Also, its chemical relationship to tyrosine, and to adrenaline and thyroxine, render of interest any contribution to our knowledge of its metabolism.

Ewins and Laidlaw [1910] investigated the effects of perfusing surviving organs with tyramine, and found that, in the case of the liver, the amine was transformed quantitatively into the corresponding *p*-hydroxyphenylacetic acid. They then attempted to isolate this acid from liver extracts after a period of incubation with tyramine, but failed to do so. Using the heart, they failed to find either tyramine or the acid after perfusion, and they believed that the benzene ring was broken down.

Work has also been done on tyramine in connection with the enzyme tyrosinase, which will utilise as its substrate aromatic compounds having a hydroxy-group in the *para*-position [Bertrand, 1907]. It attacks tyramine and produces a pigmented substance, probably a melanin.

In view of the paucity of studies on the catabolism of tyramine, and the improved methods now available for the detection of oxidative processes, it was decided to investigate the effect of the addition of tyramine on the oxygen uptake of various tissues. The Barcroft differential manometer was used, and it has been found that, with liver extracts, an oxidation of tyramine occurs. The oxygen uptake corresponds exactly to the absorption of one atom of oxygen per molecule of tyramine. Further study showed that this reaction involves not only oxidation, but also deamination, but that only half the maximum possible amount of nitrogen is evolved as ammonia. That is, one molecule of ammonia is formed from every two molecules of tyramine.

As will be shown in the experimental section, there are certain facts which clearly distinguish this system from tyrosinase. No pigment is produced, and tyrosine and *p*-cresol are not attacked. The addition of *M*/500 KCN has no effect upon the oxygen uptake, whereas tyrosinase is known to be extremely sensitive to cyanide [Lehmann, 1909]. It is therefore believed that this enzyme system has not hitherto been investigated. A preliminary account of its properties is given below, and further work is in progress.

Experimental.

Preparation of the enzyme. The livers of rat, rabbit, sheep, pig, ox and dog were found to be capable of oxidising tyramine, but that of the rabbit is especially active. Rabbit liver was therefore used in the experiments described below. The rabbit was killed by bleeding and the liver removed and finely minced. The minced liver was ground with washed sand, diluted with an equal volume of water and squeezed through muslin. The resulting liquid was very active and would keep for several days on ice, and for most experiments 1 cc. of it was used in each Barcroft bottle. This amount when fresh will oxidise 2 mg. of tyramine in about 3 hours at 15°, but it slowly loses its activity.

A cell-free solution of the enzyme was prepared by the addition of kaolin to the liver extract and adjusting to p_H 6·5. The enzyme is adsorbed by the kaolin, which is centrifuged and washed. It is then removed from the kaolin by dilute soda (p_H 8·0), and again centrifuged. The solution now contains the enzyme in a very active form, but it is unfortunately very unstable, and will not keep, even at 0°, for more than a few hours. No method has yet been found for preventing this destruction, which is probably due to a lack of the protective colloids present in the original extract. For certain of the following experiments minced liver was washed two or three times with distilled water, the liquid squeezed out through muslin, and the tissue, when required for use, was suspended in buffer or water. Difficulty was experienced in obtaining by this method a uniformly active preparation, as part of the enzyme is washed out into the water. Therefore the method of preparation first described was used for most experiments.

Preparation of tyramine. The process used was that of Johnson and Daschavsky [1925] as modified by Abderhalden and Gebelein [1926]. Briefly, tyrosine is heated to 240° in the presence of diphenylamine as a catalyst. Carbon dioxide is evolved, and the tyrosine gradually dissolves, until a clear liquid is obtained. After cooling, the tyramine may be separated from the diphenylamine by washing with ether, in which the former is almost insoluble. The crude tyramine may be converted into the hydrochloride, but was usually recrystallised directly from boiling xylene. The yield is very good, being about 90 % of the theoretical.

Properties of the system. The Barcroft apparatus was set up with 1 cc. of enzyme preparation and 1 cc. of buffer in each bottle, plus 1 cc. water in bottle (1), and 1 cc. tyramine solution in bottle (2). Phosphate buffer, p_H 7·3,

was used unless otherwise stated. The tyramine solution was made up fresh each day, so that 0·5 or 1 cc. of the solution contained 2 or 3 mg. of tyramine. Control experiments were done without tyramine and also with tyramine alone.

The oxygen uptake is very rapid at the beginning of the reaction. For this reason, the apparatus was shaken in a bath at room temperature rather than at 37°, for the reaction is then slower and the errors consequently smaller. The whole reaction is complete in about 4 hours. Towards the end the oxygen uptake becomes slow and finally ceases, this being the typical curve to be expected from an enzyme system. The amount of oxygen absorbed under these conditions is from 85 to 90 % of the theoretical, if one molecule of tyramine

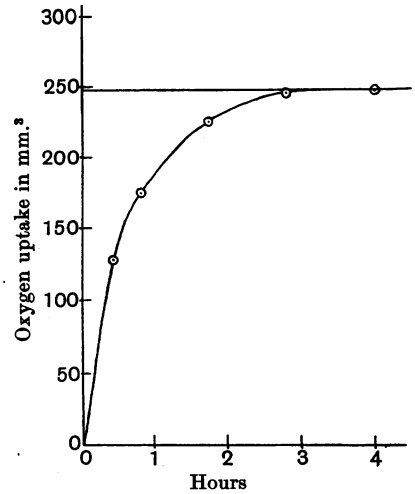

Fig. 1. The oxidation of tyramine by liver.
Theoretical oxygen uptake for 3 mg. tyramine=245 mm.³

takes up one atom of oxygen. In these experiments, however, an error always occurs when the tyramine is added to the liver extract and the apparatus has to be shaken while open to the air to allow temperature equilibrium to be reached. This error was eliminated by the use of small jars in which the tyramine may be put inside the Barcroft bottles and then upset into the liver extract when the apparatus is closed. In this way, the oxygen uptake obtained corresponds exactly to the theoretical. An experiment is given. All figures are corrected to normal pressure and temperature for the dry gas.

The enzyme system is thermolabile, liver extract after boiling for 3 minutes showing no oxygen uptake whatever with tyramine. The system is unaffected by the presence of cyanide at a concentration of $M/500$, although the oxygen uptake of liver extract alone is approximately two-thirds inhibited. Other workers have shown that a concentration of $M/1000$ KCN is sufficient completely to inhibit systems such as succinoxidase [Szent-Györgyi, 1924] and Warburg [1921, 1923, 1, 2] has put forward a theory that all oxidations

occurring in the cell are dependent upon the presence of traces of iron to "activate" the oxygen. Dixon [1925] has shown that the xanthine oxidase forms an exception to this hypothesis, and the tyramine oxidase is another example of the type of oxidation which is independent of traces of iron.

Exp. 1. Four Barcroft apparatus were used, and each bottle contained 1 cc. liver extract and 0·9 cc. buffer. Bottle (1) of each apparatus contained 0·5 cc. water, and bottle (2) contained 0·5 cc. (*i.e.* 2 mg.) tyramine solution, which was enclosed in a small jar. Apparatus *V* and *W* were controls, and contained 0·6 cc. water in both sides, whereas *X* and *Y* contained 0·6 cc. *M*/100 KCN instead. As no difference is obtained in the curves when cyanide is present, only isolated points are given. The figures (Table I) represent the oxygen uptake in mm.[3]

Table I.

Time (hours)	*V* (no KCN)	*W* (no KCN)	*X* (KCN *M*/500)	*Y* (KCN *M*/500)
0·5	107	99	111	113
1	149	138	142	148
2	161	155	160	160
3	164	162	167	163

The theoretical oxygen uptake for 2 mg. tyramine is 163 mm.[3]

Other organs were now prepared in the same way as the liver, and kidney, lung, suprarenals, skeletal muscle and heart were tested. Of these all were negative except the kidney, which showed a slight uptake with tyramine. It was expected that extract of heart would show an oxidation, for in the experiments of Ewins and Laidlaw the oxidation of the tyramine proceeded further with heart perfusions than with the liver. Several different preparations of heart were tried, but no oxidation could be obtained, so that it is evident that the mechanism of the oxidation is different in the case of the heart from that of the liver, in that in the former the system cannot be extracted and is probably dependent upon the intact organ structure.

Experiments were now carried out on the influence of different hydrogen ion concentrations on the activity of the liver tyramine oxidase. The liver extracts were taken to the required p_H by the addition of acid or alkali, and then suitably buffered, acetate, phosphate or borate buffer being used. Control experiments showed that the oxygen uptake of the tyramine was not influenced by any of these anions.

In all cases, a second portion of the extract was similarly treated, but was readjusted to p_H 7·3 before adding the buffer, in order to show whether the system was actually destroyed or merely inhibited by the treatment. The activity was determined by the amount of oxygen taken up during the first hour. It will be seen that the enzyme is very much more active in alkaline solutions than in neutral, and reaches its optimum at p_H 10·0, showing a sudden diminution in activity between p_H 10·0 and 11·0. On the acid side, it is inhibited, but not destroyed, at p_H 4·4 and it is completely destroyed in very alkaline solutions (p_H 11·5). Control experiments were tried with tyramine alone, but no autoxidation was found to occur.

In view of the possibility that direct deamination might occur during this reaction, accompanying or independent of the oxidative process, ammonia estimations were done on the contents of the Barcroft bottles after the oxygen uptake had ceased. Such a deamination was found to occur, as the bottles with liver extract and tyramine contained very much more ammonia than the controls which contained liver extract and water. When washed liver or the kaolin preparation was used, the ammonia content of the controls was very small, but the content of fresh liver varied very much; nevertheless, the same amount of extra ammonia was present after the oxidation of the tyramine in every case. A vacuum distillation method was adopted for the estimation of the ammonia. The contents of the Barcroft bottle were washed out into the distilling flask, and 5 cc. 20 % sodium hydroxide were added. The ammonia was

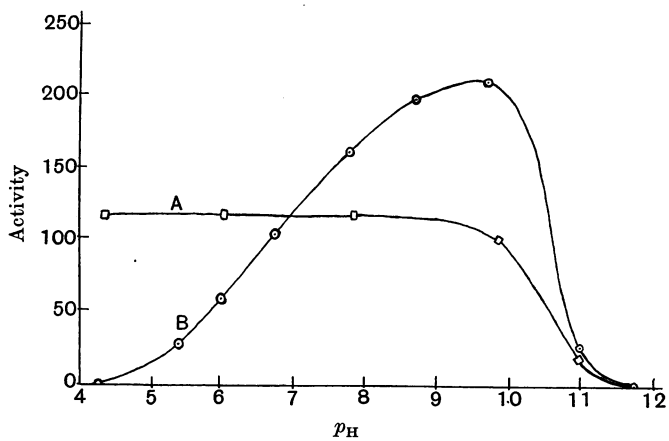

Fig. 2. The influence of hydrogen ion concentration on the activity of the tyramine oxidase system.

A = Activity of enzyme at p_H 7·3 after being subjected for 5 mins. to various hydrogen ion concentrations.
B = Activity of enzyme at different hydrogen ion concentrations.

distilled into 3 cc. $N/10$ HCl for 15 minutes at 40°. The distillate was diluted and nesslerised, one-tenth of the final volume of Nessler's reagent being used, and made up to 25 or 100 cc., the smaller volume being used for the washed liver or kaolin preparation controls, which contained very little ammonia. The colour of the nesslerised solution was compared with that of a standard in a colorimeter. No difficulty was experienced with the process of nesslerisation, and the resulting solutions were always crystal clear. Control experiments were done on tyramine alone, to show that it was not deaminised by distillation under these conditions.

It was found that the ammonia given off was only half that which is theoretically possible. This was at first thought to be due to a loss of ammonia from the Barcroft bottles, as the p_H of the reaction mixture, 7·3, was slightly on the alkaline side of neutrality. To eliminate this possibility a new apparatus was set up. Three small wide-necked bottles were tightly corked and con-

nected together with rubber and glass tubing, so that a stream of air could be sucked through them. The entry tube of each bottle reached to the bottom. The first bottle contained dilute sulphuric acid to wash the air, the second the buffered liver extract and tyramine, and the third *N*/10 HCl. The third bottle was connected to the pump, and air was slowly sucked through for about 5 hours, so that the oxidation of the tyramine should be complete. A control set of bottles was set up at the same time, in which the tyramine was replaced by water, and connected by a T-piece and taps to the same pump. The rate of the air current could thus be regulated so that it was the same for each set of bottles.

The ammonia distillations were done on the contents of the second bottle, and the liquid in the third bottle was washed out into the distillate before nesslerisation. The ammonia estimated was again found to be just half that which was expected, and it was demonstrated that the hydrochloric acid bottle contained only traces of ammonia at the end of the experiment. In view of the possibility that the presence of liver extract might alter the amount of ammonia that could be estimated, for instance by the conversion of some of the ammonia into urea, experiments were done in which a known amount of ammonium sulphate was added to the liver extract. After incubation, the ammonia was estimated as before, and the whole amount of the added ammonia was recovered. Ammonia estimations were also carried out on the heart extracts and tyramine after incubation, as it was thought possible that deamination might occur without oxidation, but the amount of ammonia was identical with that found in the controls. Table II shows these results.

Table II. *The deamination of tyramine by certain tissues.*

Tissue	Tyramine added (mg.)	Time of incubation in hours at 15°	Total NH_3-N estimated (mg.)	NH_3-N evolved from tyramine (mg.)
(1) 2 cc. liver extract	None	5	0·048	—
„	3	5	0·195	0·147
(2) 2 cc. washed liver	None	6	0·011	—
„	3	6	0·166	0·155
(3) 2 cc. washed liver	None	6	0·014	—
„	3	6	0·172	0·158
(4) 2 cc. liver extract	None	5	0·130	—
„	2	5	0·230	0·100
(5) 2 cc. kaolin preparation	None	5	0·044	—
„	2	5	0·148	0·104
(6) 2 cc. kaolin preparation	None	5	0·046	—
„	2	5	0·148	0·102
(7) 2 cc. heart extract	None	6	0·09	—
„	3	6	0·08	None

Control experiments.

	NH_3-N added as $(NH_4)_2SO_4$ (mg.)			NH_3-N recovered (mg.)
(8) 2 cc. liver extract	None	5	0·190	—
„	0·100	5	0·295	0·105
(9) 2 cc. liver extract	None	5	0·195	—
„	0·100	5	0·301	0·106

Liver extract was now incubated anaerobically in Thunberg tubes for several hours, 3 cc. buffered extract and 1 cc. tyramine solution or 1 cc. water were added and the tubes evacuated. After about 5 hours ammonia estimations were made and it was found that there was no increase of ammonia in those tubes which contained tyramine. This shows that the deamination is connected with the oxidative process and does not occur without the latter.

Further experiments were now carried out to show the relationship of the amount of ammonia produced at various stages to the oxygen absorption during the reaction. This relation is shown graphically in Fig. 3.

It will be seen that the deamination is proportional to the oxygen uptake, but that it ceases when only half the nitrogen of the tyramine molecule has been liberated as ammonia. When the oxidation is complete the deamination also stops, and no more ammonia is evolved.

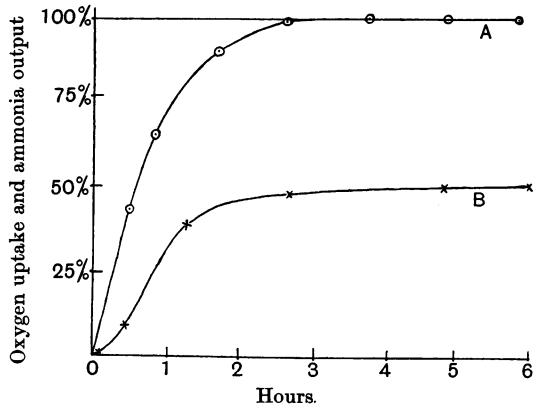

Fig. 3. Relationship of the deamination to the oxidation of tyramine by liver.

Values given as percentages and (*a*) the theoretical oxygen uptake as calculated for 1 atom per molecule of tyramine (A), and (*b*) the theoretical maximum ammonia-nitrogen (B).

The Thunberg technique was now employed to see whether the presence of tyramine would accelerate the reduction of methylene blue by liver extracts. A great many experiments were carried out, but no considerable acceleration could be demonstrated with any of the liver preparations used. The explanation of this negative result may be that the reduction potential of the system is too low to reduce methylene blue, but that a dye having a higher r_H (that is a lower reduction potential) than methylene blue might be reducible. Therefore certain dyes (nos. 4, 6 and 9 in the Mansfield Clark series of r_H indicators, methylene blue being no. 3 *a*) were made up in solutions equivalent to methylene blue, 1/5000, and put up in vacuum tubes with liver extract and tyramine. The liver preparation was used, but dyes nos. 6 and 9 were reduced so rapidly by the extract alone that the experiment was not conclusive, and in the case of no. 4, no acceleration could be detected in the presence of tyramine. *m*-Dinitrobenzene was also tested as a possible hydrogen acceptor. The solid was finely ground, and about 0·2 g. was added to each Thunberg

tube. 2 cc. enzyme preparation, 2 cc. buffer, and 1 cc. tyramine solution or 1 cc. water were added and the tubes evacuated. After incubation for a few hours the tubes were opened and made alkaline by the addition of sodium hydroxide; reduction is indicated by the appearance of a dark red colour, but this was not deeper in the tyramine tubes than in the controls.

Experiments were now performed to see whether the production of hydrogen peroxide during the reaction could be demonstrated. The technique was that used by Thurlow [1925] and is based on the fact that hydrogen peroxide in presence of milk peroxidase oxidises nitrite to nitrate; the residual nitrite is estimated by acidifying and adding the Griess-Ilosvay reagent.

Exp. 2. Three flasks were set up, each containing 2 cc. liver extract in buffer, p_H 7·3, plus 3 cc. milk peroxidase solution, and $NaNO_2$ to make the final concentration $N/1000$; to flask no. 1 was added 1 cc. water, to flask no. 2 1 cc. tyramine solution (3 mg.). Nothing was added to the third flask. The flasks were then incubated at room temperature for 5 hours. 1 cc. tyramine solution (3 mg.) was then added to flask no. 3, and immediately 2 cc. acetic acid and 2 cc. Griess-Ilosvay reagent were added to each flask.

Flask	Colour
1	+ + + + +
2	+ +
3	+ + + + +

Liver tissue alone reduces nitrate to nitrite [Bernheim and Dixon, 1928], so that a complete oxidation of the nitrite could not occur. Flask no. 3 in the experiment was put up to show that the diminution in the amount of nitrite was not due to the interaction of the amine group of the tyramine molecule and the nitrite after acidification. A concentrated solution of milk peroxidase was added to compete with the catalase present in the extract for the available hydrogen peroxide. The above experiment is typical of many.

Many other substances have been tried as possible substrates, including tyrosine, phenylalanine, dihydroxyphenylalanine, *p*-cresol, phenol, aniline and adrenaline, but these were found to be unattacked. *p*-Aminophenol is slowly autoxidisable at p_H 7·3 and the addition of liver extract causes a slightly more rapid oxygen uptake. However no deamination occurs and it is not known whether the acceleration is due to the same system as the tyramine oxidation.

A few preliminary experiments have been performed with phenylethylamine. When this is neutralised and added to the Barcroft apparatus containing liver extract it shows an oxygen absorption equivalent to one atom per molecule, which is unaffected by the addition of $M/500$ KCN. The production of hydrogen peroxide during the reaction can be demonstrated and marked deamination occurs. Thus it seems probable that the system responsible for this oxidation is identical with the tyramine oxidase; further work is in progress on this point.

The liver extract as used contains also other oxidising enzymes and certain

substances, such as *p*-phenylenediamine, are oxidised by it, but these are also oxidised by other tissues [Battelli and Stern, 1912], which have no effect on tyramine.

Certain substances were now added to the tyramine oxidising system, with a view to the possible inhibition of the reaction. The substances mentioned above, also ammonium sulphate, produced no effect. *p*-Hydroxyphenylacetic acid, the end-product in Ewins and Laidlaw's experiments, is also inactive in this respect. However, it was noticed that the further addition of tyramine to the reaction mixture in the Barcroft apparatus, after the original uptake had ceased, resulted in a renewed uptake which was slower than the first, but which eventually reached nearly the theoretical value. Further oxidation occurred after a third addition of tyramine, but the uptake was now greatly slowed (Fig. 4).

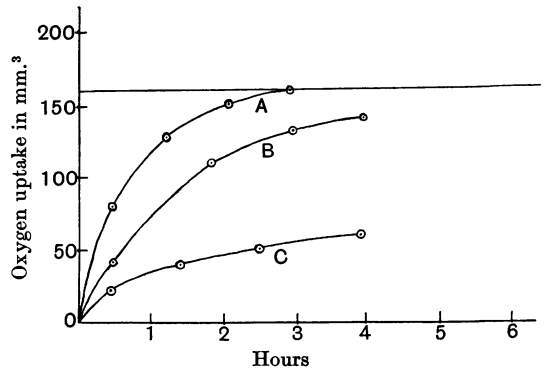

Fig. 4. The effect of successive additions of tyramine to liver extract.

Curves A, B and C represent respectively the oxygen uptake after the first, second and third additions of 2 mg. tyramine.

These facts suggest that the inhibition is due to an increasing concentration of the end-products of the reaction, and this is made still more probable by the following experiment. A mixture of liver extract and tyramine which has been incubated for several hours is deproteinised by the addition of "colloidal iron" and sodium sulphate, in the proportions suggested by Hiller and van Slyke [1922]. The clear filtrate is evaporated to dryness on a water-bath and a portion of the residue, about 10 mg., is added to both sides of a Barcroft apparatus, which contains liver extract and tyramine as usual. The result is a marked inhibition, as shown in the curve (Fig. 5).

Control experiments show that this effect is not due to an inhibition of the oxygen uptake of the liver preparation alone, and it is probably due to the increased concentration of the end-products.

The properties of the substance obtained as above by the removal of the protein from the reaction mixture were studied. An aldehyde seemed a possible product, though this, if formed, would probably undergo a Cannizzaro reaction. The presence of an aldehyde could not be demonstrated by any of the ordinary

chemical tests, and in view of the fact that the reaction does not involve complete deamination, the quantitative production of the corresponding aldehyde, or *p*-hydroxyphenylacetic acid, appears improbable. Further experiments are in progress on the amino-nitrogen content of the deproteinised residue. Large scale experiments are also in preparation, by means of which it is hoped to determine the nature of the final product of the reaction.

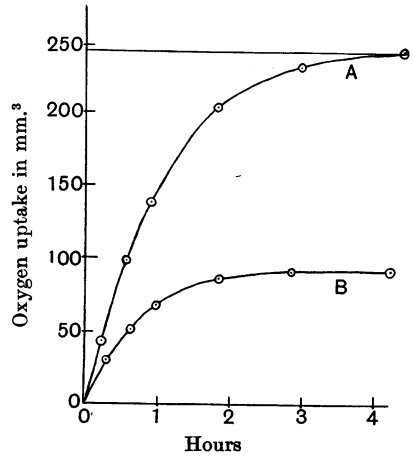

Fig. 5. The effect of addition of deproteinised residue on the oxygen uptake of the tyramine oxidase system.

A=control. B=plus 10 mg. solid residue.

Discussion.

The oxidation of tyramine by liver, as discovered by Ewins and Laidlaw, which has been shown to be due to a definite enzyme system, may be of signi ficance in three possible ways.

Firstly, the system may be protective and present for the purpose of effecting the rapid detoxication of excessive amounts of tyramine absorbed from the intestine. In this connection it is worthy of note that rabbit liver has been found to contain the most rapid and efficient tyramine-oxidising system, and this may be correlated with the fact that rabbits have a very large caecum, which probably harbours an unusually large number of bacteria, and these may produce more tyramine than those in the intestine of a carnivore.

Secondly, it is possible, though unlikely, that tyramine or some nearly related compound is formed during the normal catabolism of tyrosine and then may be oxidised by this enzyme. A decarboxylating mechanism for tyrosine is however not yet known, and such a process would involve the postulation of an entirely new mode of catabolism for the amino-acids.

Thirdly, it is possible that this oxidative deamination is connected with the synthesis of some substance which is necessary for the animal body. There are certain facts, stated in the experimental section, which lead to the

idea that the end-product of the reaction is not the simple acid, *p*-hydroxyphenylacetic, isolated from perfusion fluids by Ewins and Laidlaw. Particularly, they failed to find the acid after incubation of liver extracts and tyramine, and the incomplete deamination which occurs suggests that the final product cannot be free from nitrogen. Possibly, two molecules of tyramine condense, forming a dipeptide linkage, or, two nuclei may join together by means of an ether linkage, and the nitrogen of one molecule may remain unattacked as an amine side chain.

There are several very interesting properties exhibited by the tyramine oxidase system. It resembles the xanthine oxidase in being unaffected by the addition of cyanide, and thus forms another exception to Warburg's statement that no direct oxidation can proceed in which atmospheric oxygen is not "activated" by iron. The production of hydrogen peroxide during the reaction seems to show that molecular oxygen acts as a direct acceptor of hydrogen in the system. Whether or not any possible correlation exists between these two facts is still uncertain. The possible significance of the occurrence of hydrogen peroxide during the process of an oxidation has been fully discussed by Thurlow.

On the other hand, it differs from the above system in that, whereas the latter readily reduces methylene blue, the tyramine oxidase system apparently cannot utilise this dye as a hydrogen acceptor in the place of atmospheric oxygen. In this property it resembles the "aerobic oxidases," tyrosinase and the phenolases. Whether this difference between the two classes of enzymes is due to a fundamental difference in the mechanism of their action, or is due to our lack of knowledge of the oxidation-reduction potentials concerned, is at present undecided. The tyramine oxidase system, however, occupies a unique position and cannot as yet be classed with any of the known types of enzymes. The significance of the system cannot be properly discussed until the end-products of the reaction have been identified.

Summary.

1. Using the Barcroft technique, an oxidation of tyramine (*p*-hydroxyphenylethylamine) has been shown to take place by means of an enzyme contained in extracts of liver. The oxygen uptake corresponds to the absorption of one atom of oxygen per molecule of tyramine. Methylene blue, however, is not reduced.

2. It has been shown that deamination occurs simultaneously with the oxidation, but only half the nitrogen present in the tyramine molecule can be accounted for as ammonia.

3. The enzyme can be obtained in a cell-free solution.

4. The oxidation is not affected by the addition of $M/500$ KCN.

5. The production of hydrogen peroxide during the reaction has been demonstrated.

6. The influence of hydrogen ion concentration on the system has been studied, and the optimum reaction has been shown to be at p_H 10·0. The enzyme is inactive, but not destroyed, at p_H 4·4, but destruction occurs at p_H 11·5.

7. Phenylethylamine and possibly also *p*-aminophenol are oxidised in the presence of liver extracts, but tyrosine and many other substances are unattacked. The system produces no pigment and in many ways is shown to be distinct from tyrosinase.

8. The possible significance of the enzyme system is discussed.

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