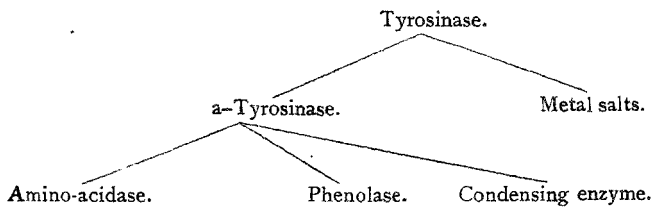
STUDIES IN ENZYME ACTION. PART IV.

Tyrosinase I.

By D. Narayanamurti and C. V. Ramaswami Ayyar.

The enzyme tyrosinase distributed among plants and animals was discovered by Bourquelot and Bertrand (*J. Pharm. Chim.*, 1896, **3**, 177; *Bull. Soc. Mycol.*, 1896, **12**, 17) and has engaged the attention of numerous biochemists; it is able to oxidise tyrosine in presence of atmospheric oxygen. That other oxidising enzymes have no action on tyrosine was first demonstrated by Bertrand who showed that the oxidases, phenolase and tyrosinase could be separated from one another. Gessard noticed its presence in *B. Pyocyaneus* and extracted the enzyme from fungi with glycerine. Russula tyrosinase was used by Harley (*J. Pharm. Chim.*, 1899, **9**, 225 & 424; 1900, **11**, 172) to test for tyrosine in digestion mixtures. Gonnerman (*Chem. Ztg.*, 1916, **40**, 127) isolated active preparations from the potato. Consequently the enzyme most studied has been prepared either from Russula or the potato.

Gessard (*Compt. rend.*, 1900, **130**, 1327) found that the red oxidation product formed by the action of tyrosinase on tyrosine is quickly transformed into melanin by salts of alkaline earth metals and of heavy metals. Though it is quite probable that salts act by coagulating the colloidal melanin formed, it is claimed by Haehn (*Biochem. Z.*, 1920, **105**, 169; *Ber.*, 1919, **52**, 2029; *Fermentforschung*, 1921, **4**, 301) that the enzyme is quite inactive in absence of salts, and he ascribes to these the function of a co-ferment. He believes that the enzyme is a mixture of components which can be represented thus :—



Similar views have been held by Bach, Folpmers (*Biochem. Z.*, 1016, **78**, 180), Onslow (*Biochem. J.*, 1923, **17**, 216) and others.

Raper and collaborators (*Biochem. J.*, 1923, **17**, 454; 1925, **19**, 84, 92; 1926) have elucidated the chemical transformations involved; they recognised three distinct stages in the formation of melanin. In the first stage tyrosinase produces a red substance from tyrosine, only in the presence of oxygen. 'The red substance becomes spontaneously colourless and is then oxidised to melanin. These last two processes take place in the absence of tyrosinase, but may be accelerated by it or other oxidases found in the potato juice.' They have also shown that there are no grounds for assuming that deamination occurs when tyrosinase acts on tyrosine.

During an investigation on the proteins of *Dolichos lablab* it was observed that aqueous extracts of the seed darken on standing in air and this was traced to the enzyme tyrosinase. A survey of the literature showed that, apart from the work of Raper, which is mainly concerned with the chemical mechanism, no systematic and quantitative study of the enzyme has been made. Much of the previous work has been concerned with colour reactions and this might lead to erroneous conclusions, as seen for example in the section on the action of hydrogen peroxide. Moreover, the enzyme prepared from *Dolichos lablab* differs in several respects from the one obtained from the potato. A physico-chemical study of the enzyme is described in this paper.

METHODS.

Preparation of the enzyme.—The enzyme was generally prepared by extracting 180 gms. of the finely ground ungerminated seed of *Dolichos lablab* with 600 c.c. of water in presence of toluene for three days at room-temperature. After filtering through paper pulp the extract (containing a large quantity of protein) was dialysed under slight pressure in collodion bags against flowing distilled water for a week, when all the proteins are precipitated and the solution is now centrifuged to remove suspended matter, the resulting brown liquid being clear. The enzyme thus prepared, unlike the one from potato, is very stable in aqueous solution for many weeks at ice-box temperature.

Estimation of activity.—The method of Raper (*Biochem. J.*, 1923, **17**, 454) as modified by Haehn and Stern (*Biochem. Z.*, 1927, **184**, 182) was adopted, the principle being to estimate the balance of tyrosine left by bromination.

Experimental arrangement.—The reactions were conducted in an electrically regulated thermostat in which all solutions were kept initially for half an hour. The reactions took place in long-necked

round bottomed flasks provided with two-holed rubber stoppers. Through one hole passed a long glass tube reaching to the bottom of the flask and acting as the air-inlet, while the other took a very short tube ending flush with the bottom of the rubber stopper. After the enzyme solution, buffer and tyrosine had been mixed in the right proportion, 10 c.c. of toluene to every 100 c.c. of the reaction mixture were added as an antiseptic, to avoid frothing and unnecessary loss due to evaporation. The reaction mixture was now aerated with the help of an electrically driven 'Cenco' blower, the air previously passed through two wash bottles, one containing toluene and the other distilled water saturated with toluene.

EXPERIMENTAL.

Influence of the quantity of meal on extraction.—W. B. Hardy (*J. Physiol.*, 1905, **33**, 251) and Mellanby (*J. Physiol.*, 1905, **33**, 335) have shown that the well-known colloid solubility of globulins in dilute solutions of electrolytes depends on the quantity of globulin taken. The quantity of globulin going into solution for the same volume of peptiser increases with increasing quantity of the solid phase although some of the globulin is left. Their experiments clearly bring out the difference between the solubility of molecularly dispersed substances and colloid solubility. While a molecularly soluble substance has only one solubility at a given temperature and given solvent independent of the quantity of the solid phase, the peptisation of colloids is not similar. The recent work of Buzagh (*Kolloid Z.*, 1927, **211**, 169; 1927, **43**, 215; 1928, **44**, 156), Neunstein, Wo. Ostwald and his students (*Kolloid Z.*, 1927, **41**, 163; 1927, **43**, 249) has clearly shown that this relation is not confined to globulins, but that all colloids, inorganic as well as organic, exhibit the same behaviour. The theoretical aspect has been recently discussed by Wo. Ostwald (*Kolloid Z.*, 1927, **41**, 163; **43**, 249), who has shown that adsorption plays an important part in this process.

That enzymes might exhibit such a behaviour is conceivable and our experiments bring evidence in strong support of this. Varying quantities of the meal were shaken with 200 c. c. of distilled water and 5 c. c. of toluene for three hours in stoppered bottles of the same size and shape, and the extracts along with the solid residues centrifuged to remove all precipitated and suspended matter. Portions (50 c. c.) of each centrifugate were then dialysed under slight pressure in parchment thimbles of the same size in flowing distilled water. In about five days all the proteins had settled and the solutions were again centrifuged, made up to the same volume and their activities compared. The results are given in Table I and Figure I

TABLE I.

Comparison of extract activities.

The reaction mixture contained 10 c. c. acetate buffer at P_H 6·5, 25 c. c. tyrosine solution (0·25 per cent.), 10 c. c. distilled water, 5 c. c. dialysed enzyme extract and 5 c. c. toluene. The temperature was 25° and the duration of experiment, 2 hours for the extract used soon after dialysis (fresh) and 4 hours for the aged one.

Weight of meal in gms.	Activity in c. c. thiosulphate corresponding to tyrosine oxidised in 20 c. c.	
	Fresh	Aged
20	0·8	0·5
40	2·9	2·8
60	4·1	4·1
70	2·4	6·0
80	1·6	4·4
90	1·8	4·5
100	3·1	7·8
120	3·3	8·0

It is evident from the results that the quantity of enzyme-complex extracted is dependent on the quantity of the solid phase. While the investigations of Buzagh with inorganic colloids indicate that the colloid solubility increases at first with increasing quantity of the substance and then falls with further increase of the solid phase we met with two optima, their position being changed with age. This result indicates that perhaps the enzyme is a mixture of two components with different optima, or that an activator is present in the meal; such an activator must be in the colloidal condition, as indicated by our electro-dialysis and ultra-filtration experiments.

The influence of hydrogen-ion concentration.—Search in the literature showed that this factor has not been determined in the case of tyrosinase. Raper (*Biochem. J.*, 1923, **17**, 454) working with potato tyrosinase found that the activity was greater at P_H 7·0 than 6·0, and greater at P_H 8·0 than 7·0. No clear maximum has been defined. The optimum for the enzyme from *Dolichos lablab* was found to be in the neighbourhood of 6·5. It thus differs from the potato enzyme, the activity at P_H 8·0 being considerably reduced. The optimum was

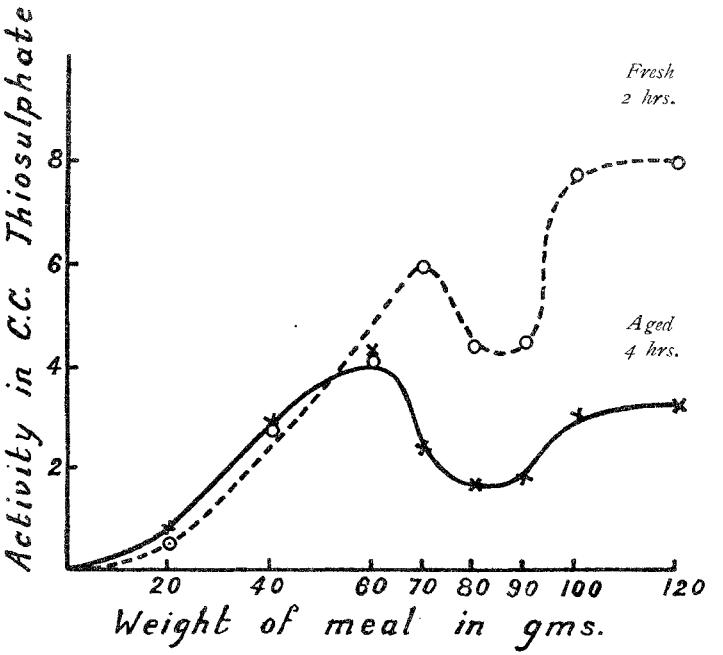

Activity in C.C. Thiosulphate
8
6
4
2
Fresh
2 hrs.
Aged
4 hrs.
20
40
60
70
80
90
100
120
Weight of meal in gms.

Fig. I

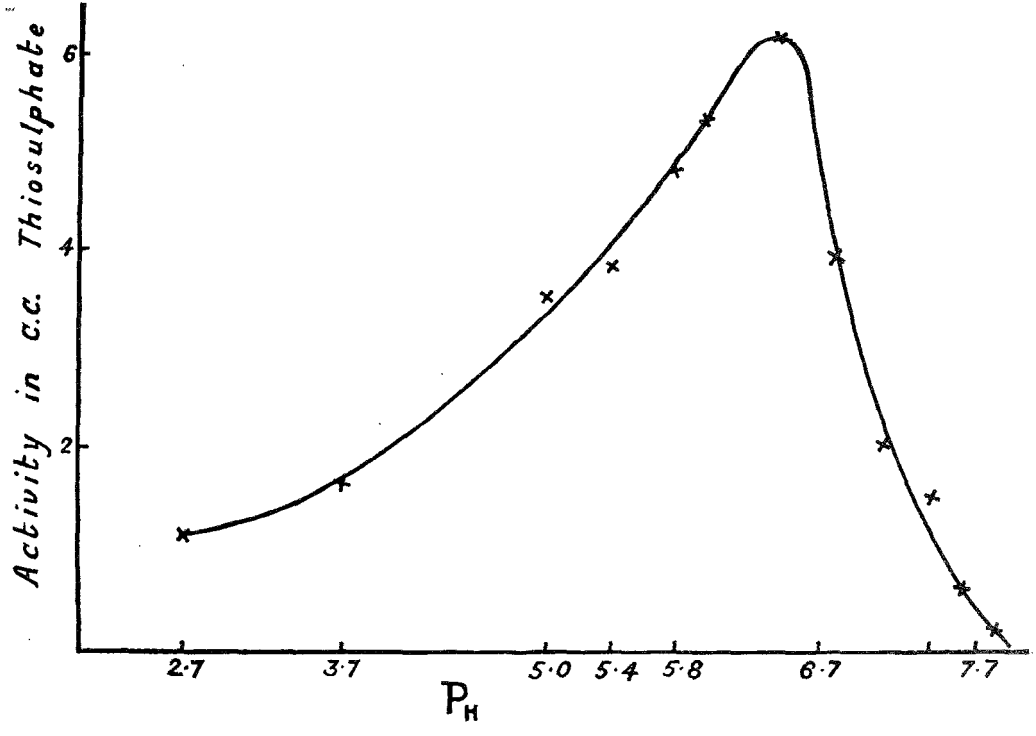


Fig. II

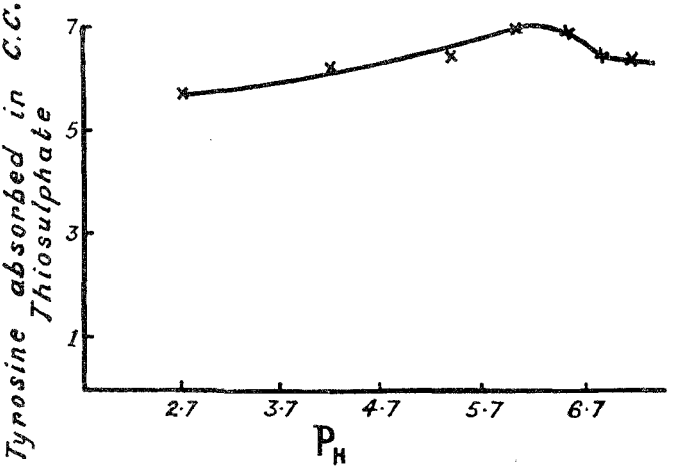


Fig. III

found by measuring the activity of the enzyme at different hydrogen-ion concentrations, from 2·7 to 6·5, in Walpole's acetate buffer and in Palitzsch's borate buffer for the range 6·7–8·0. The results are given in Table II.

TABLE II.

The reaction mixture contained 10 c.c. buffer solution, 25 c.c. tyrosine solution (0·1 per cent.), 10 c.c. distilled water, 5 c.c. dialysed enzyme extract and 5 c.c. toluene. The temperature was 30° and the duration of experiment 4 hours.

P_H	2·7	3·7	5·0	5·4	5·9	6·0	6·5	6·8
Tyrosine oxidised (c.c. thiosulphate) ...	1·1	1·6	3·5	3·8	4·8	5·3	6·1	3·9
P_H	7·1	7·4	7·6	7·8				
Tyrosine oxidised (c·c. thiosulphate) ...	2·0	1·5	0·5	0·2				

The enzyme from *Dolichos lablab* is more stable towards acidity. Raper found that the potato enzyme was inactive below P_H 4·0 and explains this on the possibility that the enzyme is bound to a protein which is precipitated at this P_H. On the other hand the enzyme occurring in *Dolichos* is active even at P_H 2·7, though the activity is considerably diminished. Another interesting fact to be noted is that we found the absorption of tyrosine by 'Norit' is also at a maximum at P_H 6·0–6·5. The results are given in Table III and Figure III.

TABLE III.

The mixture contained 20 c.c. buffer solution, 50 c.c. tyrosine solution (0·1 per cent.), 0·5 gm. 'Norit' (passed through 150 mesh sieve) and 30 c.c. distilled water. It was shaken and kept in an incubator at 32° for 24 hours to attain equilibrium, then was filtered and the tyrosine left in solution estimated in 20 c.c. samples.

P_H	2·7	4·2	5·4	6·0	6·8	7·1
Tyrosine adsorbed (c.c. thiosulphate) ...	5·7	6·2	6·4	6·9	6·4	6·4

Tyrosine is isoelectric over the range 4·5–6·5 and it is evident from our adsorption experiments and from those of Abderhalden and Fodor with several amino-acids that these are best adsorbed at their isoelectric points. The experiments indicate that oxidation is probably greater in the case of undissociated tyrosine. Experiments are also in progress to measure the adsorption of tyrosine on tyrosinase at various hydrogen-ion concentrations.

The Isoelectric Point of Tyrosinase.—This constant has not hitherto been determined and is of great theoretical importance with regard to the nature of enzyme action, whether viewed from Michaelis' electro-chemical dissociation theory or from the adsorption theory. Qualitative experiments made by us indicate that the isoelectric point of tyrosinase prepared from *Dolichos lablab* approximates to P_H 6·2. The enzyme solution (10 c.c.) was usually mixed with 5 c.c. of the buffer having the required P_H and placed in the middle chamber of a Michaelis cataphoresis apparatus of pyrex glass. The stopcocks were closed and the two side limbs filled with buffer solution (5 c.c. diluted with 10 c.c. of conductivity water). A platinum foil dipping in the buffer solution in contact with solid monosodium phosphate (Sherman, *J. Amer. Chem. Soc.*, 1924, **46**, 1711) served as anode, and copper in copper sulphate was used as cathode. The electrodes were connected to the side limbs of the U-tube by agar junctions. The current (220 volts) was switched on and the stopcocks opened; migration was allowed to proceed for 18 hours when the solution in the two limbs was tested for activity with tyrosine solution.

TABLE IV.

P_H	Activity of Solution	
	Anode	Cathode
6·5	Red in few minutes.	Very pale rose after 4 hours.
6·0	...	Reddens quickly.
5·57	...	Do.
7·36	Red in few minutes.	...

The results indicate that the isoelectric point lies between P^H 6·0–6·5. In the previous section it was shown that tyrosinase acts best at P_H 6·5 when tyrosine is least dissociated and most easily adsorbed. The experiments of Frumkin (*Nature*, 1926, **117**, 790; *Biochem. Z.*, 1927, **182**, 220) also clearly indicate that neutral molecules are most easily adsorbed near the isoelectric point of the adsorbent. Wright and Rideal (*Trans. Faraday Soc.*, 1928, **24**, 530) have recently shown that the decomposition of hydrogen peroxide at a variety of surfaces is greatest near the isoelectric points of the surfaces studied. The migration experiments on tyrosinase show that the isoelectric point is not far removed from the optimum P_H and hence tyrosinase action is a case of adsorption catalysis.

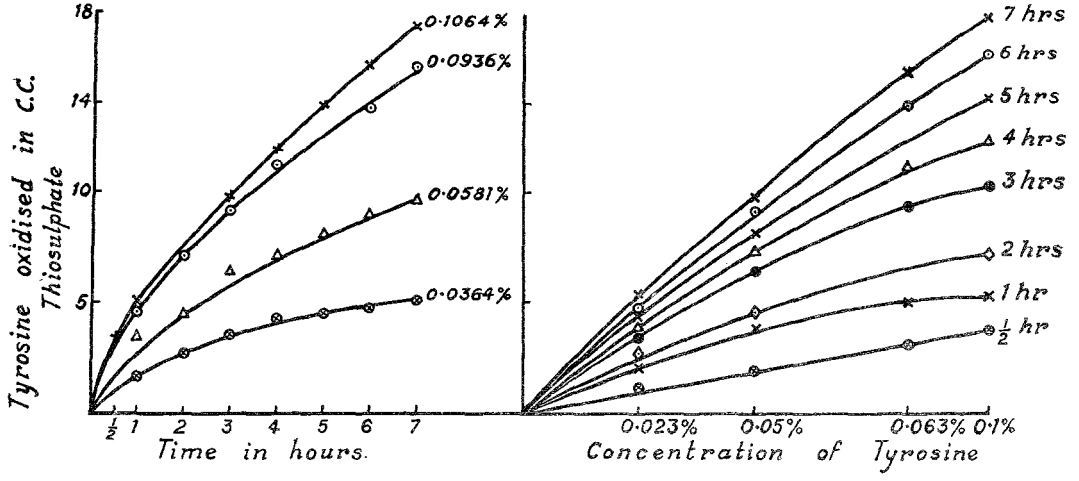

Tyrosine oxidised in C.C. Thiosulphate
18
14
10
5
0·1064%
0·0936%
0·0581%
0·0364%
½ 1 2 3 4 5 6 7
Time in hours.
7 hrs
6 hrs
5 hrs
4 hrs
3 hrs
2 hrs
1 hr
½ hr
0·023% 0·05% 0·063% 0·1%
Concentration of Tyrosine

Fig.IV

The Kinetics of tyrosinase action.

The next point of interest was to study the kinetics of tyrosinase action. The effect of substrate concentration, enzyme concentration, P_H and temperature have been investigated.

The effect of substrate concentration.—The velocity of the reaction was measured for substrate concentrations varying from 0·0364 to 0·1060 per cent. of tyrosine. As tyrosine is sparingly soluble in water higher concentrations could not be tried. The results are given in Tables V and V A and Figure IV.

TABLE V.

The mixture contained 20 c.c. of buffer solution, 20 c.c. of dialysed enzyme extract and four amounts of tyrosine, (1) 0·2128 gm. (2) 0·1871 gm. (3) 0·1162 gm. and (4) 0·0728 gm., each in 160 c.c. of water, 20 c.c. of toluene being added to each portion.

	Tyrosine oxidised in c.c thiosulphate			
Time in minutes	1	2	3	4
30	3·3	3·2	1. 8	1· 2
60	5·2	4·8	3·55	1· 8
120	7·1	...	4· 5	2· 6
180	10·0	9·1	6· 4	3· 4
240	12·0	11·1	7· 0	4·05
300	14·0	...	8· 1	4· 2
360	16·0	13·7	9· 1	4· 6
420	17·6	15·8	9· 7	5· 0
Initial thiosulphate value of the tyrosine-tyrosinase mixtures ...	23·1	20·3	12 6	7·9

TABLE V A.

	Velocity coefficient			
Time in minutes	1	2	3	4
30	0·0051	0·0057	0·0051	0·0055
60	0·0042	0·0045	0·0054	0·0043
120	0·0031	...	0·0036	0·0033
180	0·00315	0·0033	0·0039	0·0031
240	0·00305	0·0033	0·0034	0·0030
300	0·0031	...	0·0034	0·0025
360	0·0033	0·0031	0·0036	0·0024
420	0·0034	0·0036	0·0035	0·0024
Time for half change ...	3·75 hrs.	3·65 hrs.	3·6 hrs.	4 hrs.

Raper (*loc. cit.*) working with potato tyrosinase found that the reaction was unimolecular, that the velocity coefficients fluctuate during the experiment showing slight decrease during the oxidation of one-half the tyrosine followed by a larger decrease of the remainder. Our results also indicate that the reaction is unimolecular, but that the decrease in the value of the coefficient is significant in the earlier stages of the reaction and later the velocity coefficient is almost constant or fluctuates about the mean value. In the case of the 0·0364 per cent. solution however the decrease is somewhat rapid at first and then diminishes slowly but continuously. The time of half change as found from the curves also suggests a unimolecular reaction. Enzyme action can be considered as a case of adsorption catalysis, and decomposition is preceded by adsorption. At higher substrate concentrations the substance decomposed at the enzyme surface is more quickly replaced than at very low concentrations and this explains the continuous fall of the coefficient at very low concentrations. If we consider the coefficients after 3 hours, those obtained at lower concentrations, i.e., 0·0936 and 0·0581 are slightly greater than at 0·1064, again suggesting an adsorption process. In view of the small amount of tyrosine present in each sample and the number of operations involved in each experiment, the divergencies are not greater than experimental errors.

Kinetics at different enzyme concentration.—The results obtained with different enzyme concentrations are given in Tables VI and VI A and Figure V.

TABLE VI.

The mixture contained 15 c. c. of buffer solution (P_H 6·5), 100 c. c. of tyrosine solution (0·15 per cent.) with enzyme solution and water in the quantities (1) 5 and 30 c. c., (2) 15 and 20 c. c., (3) 20 and 15 c. c., (4) 25 and 10 c. c., (5) 30 and 5 c. c., toluene (15 c. c.) being added to each portion: the temperature was 25°.

Time in minutes	Tyrosine oxidised in c. c. thiosulphate				
	1	2	3	4	5
30	0·8	3·7	4·7	5·7	6·5
60	...	5·6	6·4	7·6	8·2
180	5·2	9·7	11·9	13·1	14·0
300	8·2	14·9	16·3	17·5	18·1
360	9·7	15·9	18·1	18·9	20·0
Initial thiosulphate value of the tyrosine-tyrosinase mixtures	22·7	22·6	22·7	22·9	23·2

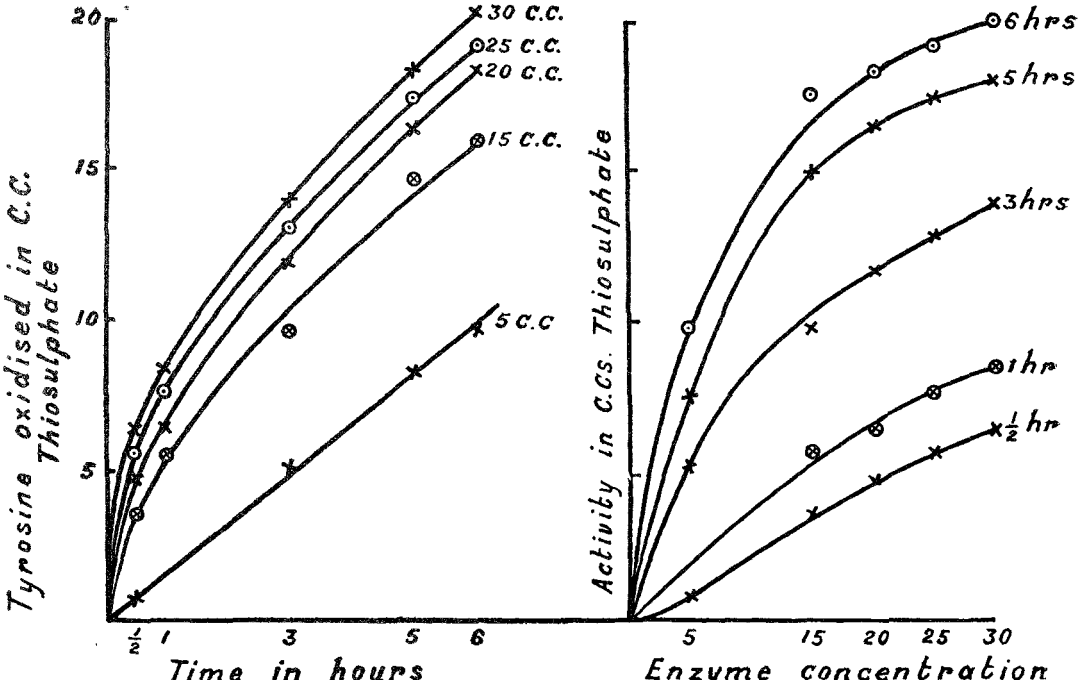

Tyrosine oxidised in C.C. Thiosulphate
20
15
10
5
30 C.C.
25 C.C.
20 C.C.
15 C.C.
5 C.C
½
1
3
5
6
Time in hours
Activity in C.Cs. Thiosulphate
6 hrs
5 hrs
3 hrs
1 hr
½ hr
5
15
20
25
30
Enzyme concentration

Fig V

TABLE VI A.

Time in minutes	Velocity coefficients				
	1	2	3	4	5
30	0·00598	0·00595	0·00772	0·00953	0·011
60	...	0·00474	0·00551	0·00671	0·00726
180	0·00144	0·00311	0·00412	0·00471	0·00513
300	0·00149	0·00359	0·00382	0·00487	0·00504
360	0·00155	0·00337	0·00443	0·00484	0·00524
Average of last three	0·00149	0·00336	0·00412	0·00481	0·00514
Time in minutes ...		30	180	360	360
$k = \frac{10\,x}{t}$ ...		0·267	0·289	0·273	0·270

While in the case of varying substrate concentration the velocity constant is almost independent of the tyrosine concentration (within the limits investigated) there is a definite increase in the value of the constant with increasing enzyme concentration. This is more marked with lower concentrations, and is to be expected because at the higher concentrations more than one-half the tyrosine is oxidised within 90 minutes. Another point to be observed is that at the lowest concentration the velocity coefficients show an increase and the coefficients for a zero order reaction fit well. It is interesting in this connection to mention that Arrhenius (*Z. Anorg. Chem.*, 1923, **36**, 456) believed that all enzyme reactions are of the zero order type. At very low enzyme concentrations, the quantity of substrate being relatively large, that part decomposed at the enzyme surface is almost immediately replaced from that in solution, so that the quantity acted upon is directly proportional to the time of action. This occurrence in tyrosinase action again suggests that adsorption plays an important role in the action of this enzyme.

Kinetics at different hydrogen-ion concentrations.—Raper has worked at three different hydrogen-ion concentrations and found the largest fall in the value of the coefficient at P_H 6·0; he believes that the acid has precipitating action on the enzyme. Our experiments at three different hydrogen-ion concentrations, 4·99, 6·52 and 7·36 give a different aspect of the phenomenon. The coefficients at P_H 4·99 show a decrease after 2 hours; at P_H 7·36 the coefficients gradually increase

and the reaction assumes an autocatalytic character. This is well illustrated by Tables VII, VII A and VII B and Figure VI.

TABLE VII.

Influence of P_H *on the kinetics.*

Composition of the reaction mixture : temperature, 25°.

P_H	0·0666 per cent. Tyrosine	Buffer	Water	Toluene	Enzyme solution
4·99	150 c. c.	20 c. c.	10 c. c.	20 c. c.	20 c. c.
6·52	do.	do.	do	do.	do.
7·36	do.	do.	do.	do.	do.

Time in minutes	Tyrosine oxidised in c.c. thiosulphate		
	P_H 4·99	P_H 6·52	P_H 7·36
30	1·0	1·9	0·6
60	2·0	3·8	1·2
120	3·6	4·4	2·4
240	5·2	6·5	5·7
300	5·5	8·0	6·7
360	5·9	...	7·5

TABLE VII A.

Time in minutes	Velocity coefficients		
	P_H 4·99	P_H 6·52	P_H 7·36
30	0·00298	0·00590	0·00176
60	0·00312	0·00612	0·00180
120	0·00305	0·00393	0·00191
240	0·00235	0·00314	0·00277
300	0·00211	0·00383	0·00283
360	0·00195	...	0·00277

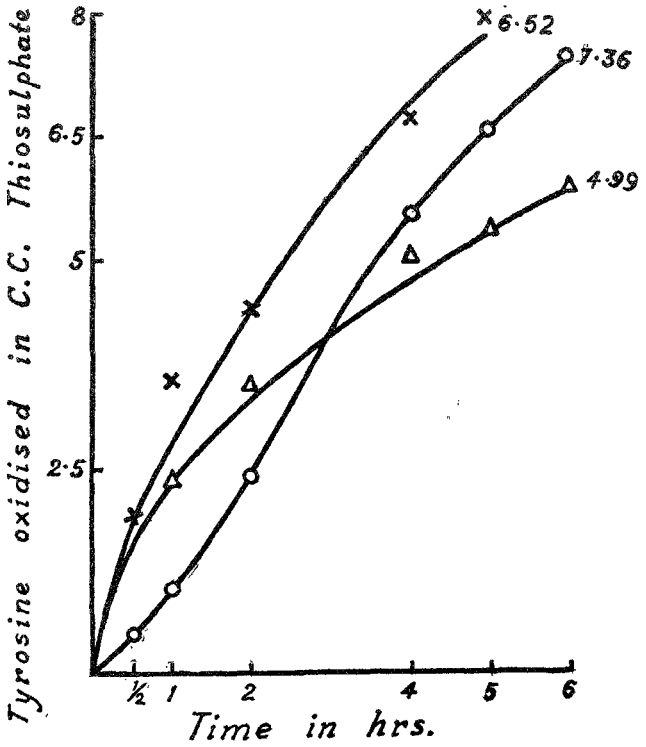

Tyrosine oxidised in C.C. Thiosulphate
8
6·5
5
2·5
6·52
7·36
4·99
½
1
2
4
5
6
Time in hrs.

Fig. VI

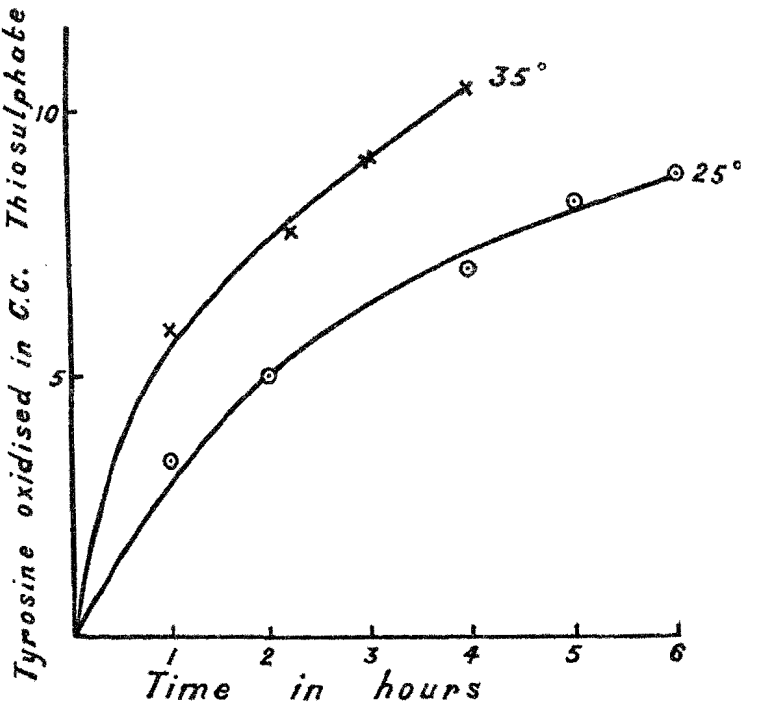

Tyrosine oxidised in C.C. Thiosulphate
10
5
35°
25°
1
2
3
4
5
6
Time in hours

Fig. VII

TABLE VII B.

Time in minutes.	30	60	120	240	300	360
$k = x/t$; P_H 7·36	0·020	0·020	0·020	0·024	0·022	0·021

The initial thiosulphate value of the reaction mixture was 11·7 c.c.

It must also be mentioned that Raper observed (with potato tyrosinase) a slight decrease at P_H 8·0 and he attributes this to the possible adsorption of the enzyme on the melanin formed.

Effect of Temperature.—As the temperature coefficient of a reaction is an important factor this was determined. The reaction was conducted at two different temperatures 25° and 35°. The results are given in Table VIII and Figure VII.

TABLE VIII.

The reaction mixture contained 100 c. c. tyrosine (0·08 per cent.), 32 c. c. buffer at P_H 6·5, 12 c. c. water, 16 c. c. enzyme solution and 16 c. c. toluene.

Time in minutes ...	60	120	135	180	240	300	360
Tyrosine oxidised 25°	3·4	5·2	...	...	6·9	8·3	8·7
(c. c. thiosulphate) 35°	5·9	...	7·3	9·0	10·2	...	...

Initial thiosulphate value of the reaction mixture was 10·2 c. c.

Time in minutes	Velocity coefficients		
	k_{25}	k_{35}	k_{35}/k_{25}
60	0·00675	0·0147	2·13
120	0·00593	...	...
135	...	0·00984	1·66
180	...	0·00851	...
240	0·00469	0·0082	1·83
300	0·00412	...	...
360	0·00423 Average of last three 0·00435	... Average of last two 0·00835	... Average of last two 1·75

The coefficients at 35° are greater than those at 25° and the ratio k_{35}/k_{25} is approximately 2. The heat of activation calculated according to the Arrhenius equation :—

$$A = \frac{\log k_2 - \log k_1}{0{\cdot}4343} \times R \frac{T_2 T_1}{T_2 - T_1}$$

is 12,250 calories.

It is also interesting to note (from the results for 35°) that the reaction can proceed to completion for the tyrosine oxidised in 4 hours is 100 per cent.

Influence of External Factors.

Temperature.—The thermostability of the enzyme in aqueous solution was next investigated. That plant tyrosinase preparations are moderately stable to heat has been noticed by Bertrand as well as by Haehn. Equal quantities of the enzyme solution were exposed to temperatures of 65–70°, 80° and 95°, for half an hour. At the end of this period the solutions were centrifuged to remove suspended and heat-coagulated matter and their activities compared with that of an untreated solution. The results are given in Tables IX and IX A.

TABLE IX.

The reaction mixture contained 25 c. c. tyrosine (0·1 per cent.), 10 c. c. buffer at P_H 6·5, 10 c. c. water, 5 c. c. enzyme solution and 5 c. c. toluene.

No.	Exposed to	Observation
1	(Untreated)	Clear solution.
2	65–70°	Slightly opalescent.
3	80°	Coagulates; white precipitate.
4	95°	Precipitate larger than in 3.

TABLE IX A.

Exposed to	Activity in c. c. thiosulphate
(Untreated)	5·7
65–70°	5·0
80°	Light rose coloration after 4 hours
95°	No action

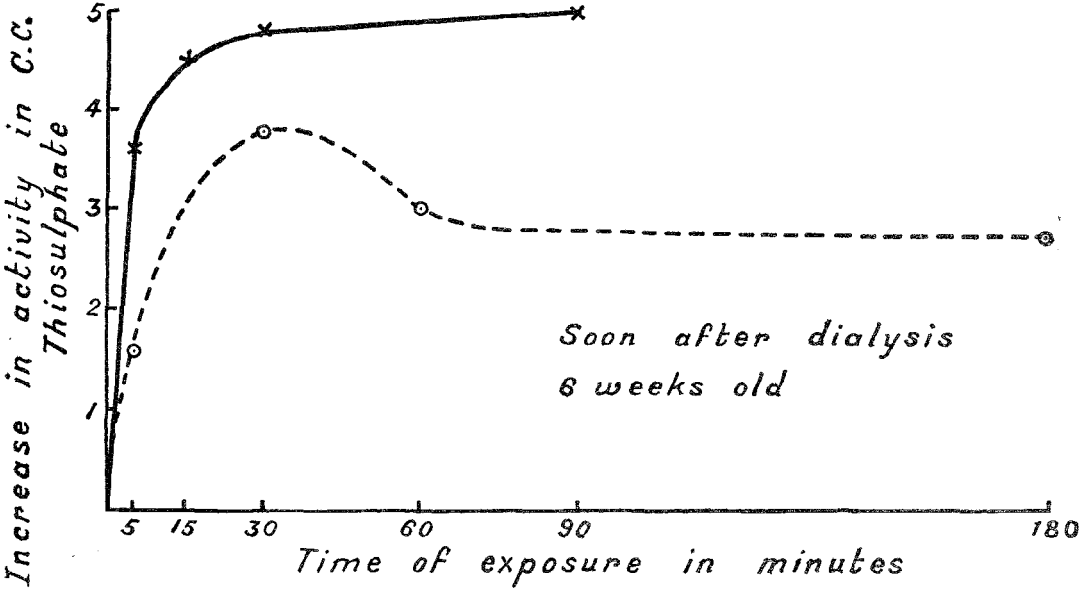

Increase in activity in C.C. Thiosulphate
5
4
3
2
1
5
15
30
60
90
180
Time of exposure in minutes
Soon after dialysis
6 weeks old

Fig. VIII

The duration of the reaction was two hours and the temperature 25°.

The results show that the enzyme is fairly stable to 70°, although its activity is slightly diminished; it is almost destroyed in 30 minutes at 80° and completely at 95°.

Action of ultra-violet light.—According to Green, Schmidt-Nielsen and Jodlbauer ultra-violet rays have a destructive action on all enzymes. Agulhon (*Compt. rend.*, 1911, **153**, 979) distinguishes the effect of the rays in presence and absence of oxygen. The effect of exposure to the rays from a quartz mercury lamp (110 volts) was tried on the *Dolichos* tyrosinase in aqueous solution, and the activity found to be increased. The results are given in Table X and Figure VIII.

TABLE X.

Time in minutes	Activity in c. c. thiosulphate	Increase in activity
0	4·4	...
5	8·0	3·6
15	8·9	4·5
30	9·2	4·8
90	9·4	5·0

TABLE XA.

Time in minutes	Activity in c. c. thiosulphate	Increase in activity
0	7·1	..
5	8·7	1·6
30	10·9	3·8
60	10·1	3·0
180	9·8	2·7

The enzyme solution was exposed to the action of light from the quartz mercury lamp in silica flasks. Table X shows results obtained with an enzyme solution exposed to the action of the rays soon after extraction and dialysis; XA the results from a solution 6 weeks old. The duration of the experiment in the first case was 3 hours and in the second case 3 hours 20 minutes. The results

clearly show an increase in activity even in the case of the aged solution, and that after reaching a maximum it slightly diminishes.

It is known that ultra-violet rays have two different biological effects, a stimulative effect and a lethal one. Stimulation is caused by rays from the limit of the visible spectrum to about 290 $\mu\mu$ (2900 A°). This corresponds to the ultra-violet component of the solar spectrum. Waves shorter than 290 $\mu\mu$ (2900 A°) exert a lethal effect.

It was observed by Lindner (*Woch. Brau.*, 1922, **39**, 166) that exposure to ultra-violet rays considerably increased the fermentation of dextrose by yeast, and that exposure of the cells in a shallower layer produced the opposite effect. Clark (*Amer. J. Physiol.*, 1922, **61**, 72; *Amer. J. Hyg.*, 1922, **2**, 322) supports the theory that action of light on organic compounds is an emission of electrons from the substance affected. Howell found that precipitation of fibrinogen by ultra-violet rays is inhibited in the presence of hematoporphyrin.

These observations suggest a possible mechanism of the action of ultra-violet rays on tyrosinase, namely, that by emission of electrons the negative charge on the enzyme particle is considerably lowered and brought to a state when it has a very small velocity of migration in an electric field. We have indicated in previous sections of this paper that tyrosinase action is to be considered as an instance of adsorption catalysis and that its activity is greatest when the enzyme has a small negative charge. The fall in the increase in activity with longer exposures is also easily explained on the basis of this theory. Cataphoretic studies are in progress to test this. Another possible explanation is the probable higher dispersion effected by the ultra-violet due to the action of some compound present as in Howell's experiment (*Archiv. inter. physiol.*, 1921, **18**, 269). A third possibility is the formation of an activator. Another point to be considered in this connection is the probable absorption of the lethal rays before they reach the enzyme. Experiments are in progress to study the effect under standardised conditions, using pure enzyme preparations. The effect of different parts of the mercury arc spectrum, added impurities, salts, P_H, etc. are being studied.

Action of Hydrogen Peroxide.—Gessard found that hydrogen peroxide activates tyrosinase and Bach made a similar observation with potato tyrosinase; both Staub and Abderhalden found inactivation. This is of theoretical importance, for according to Oppenheimer, if Gessard's finding is confirmed, then the probability that tyrosinase is a dehydrase becomes greater. Our results are given in Table XI.

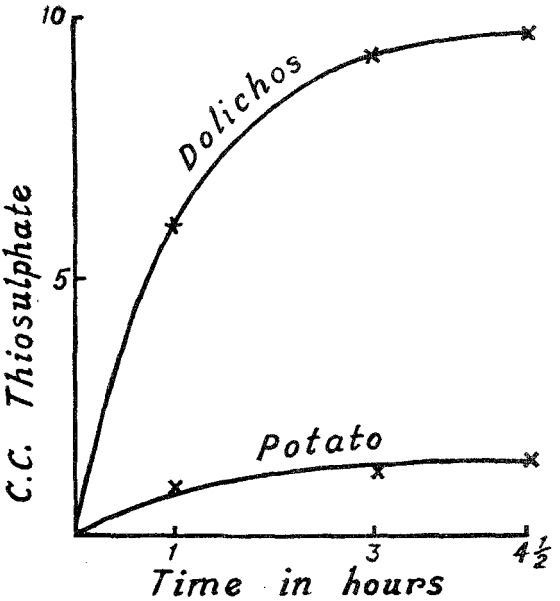


Fig. IX

TABLE XI.

Peroxide per cent.	Colour.	Activity in c.c. thiosulphate			
		Unaerated		Aerated	
		Immediate	3 hours	Immediate	3 hours
0	Red after a few minutes..	0	4·6	0	8·0
0·2	Deep red immediately ...	2·5	2·5	2·5	2·5
0·02	Deep red in a few seconds.	1·5	1·5	1·4	4·2
0·002	Red in a few seconds ...	1·5	1·5	1·5	6 3
0·0002	Red in a few minutes ...	1·0	2·7	1·0	8·9

Two series were run, one aerated and the other unaerated. No activation was observed in the unaerated samples. If readings were taken immediately after adding the hydrogen peroxide and enzyme, activation was observed, but after three hours there was practically no increase in the amount of tyrosine oxidised. In the case of aerated samples slight activation was observed at the lowest concentration. Perhaps two effects have to be taken into account, activation of some part of the reaction and destruction of the enzyme by the hydrogen peroxide.

Comparison with the enzyme prepared from Potato.—Since much of the previous work involved tyrosinase prepared from the potato, the activity of this was compared with that of the enzyme prepared from *Dolichos*. The best potatoes available in the market were minced and the juice obtained with a Buchner press at 1·5 tons, centrifuged to remove starch and dialysed in collodion bags under slight pressure against flowing distilled water. The activity of the dialysed solution was then compared with that of an enzyme preparation obtained from *Dolichos lablab* under identical conditions, the solid content of the two solutions being the same. The results are given in Table XII and Figure IX.

TABLE XII.

Time in minutes	Tyrosine oxidised in c.c. thiosulphate	
	Dolichos	Potato
60	6·0	1·0
180	9·2	1·2
270	9·5	1·4

The activity of the enzyme prepared from the potato is very much lower than that obtained from *Dolichos* under the same conditions. Haehn *(Biochem. Z.*, 1920, **105**, 169) has stated that potato tyrosinase loses its activity on dialysis and that addition of boiled potato-juice restores the activity. We did not notice this and the undialysed juice was not more active than the dialysed one. Raper *(Biochem. J.*, 1923, **17**, 454) has drawn attention to the fact that the activator is present only in the juice of fresh potatoes, and the potatoes we used were not fresh. According to Abderhalden *(Fermentforschung*, 1926, **8**, 479) the enzyme prepared from the potato is of poor activity, is stable only for a short period and is rich in ballast material and impurities. On the other hand our preparations from *Dolichos* are very active, and preserve their activity in aqueous solution over a considerable length of time.

Purification of the enzyme.—Apart from ordinary dialysis and precipitation with alcohol no purification study has been attempted by earlier workers. The increase in activity obtained with diastase (Narayanamurti and Norris, *J. Ind. Inst. Sc.*, 1928, **11A**, 134) by electrodialysis led us to try this method, which ought to be valuable in the study of tyrosinase as Haehn has claimed *(Biochem. Z.*, 1920, **105**, 169) that tyrosinase could be separated into two components by dialysis as well as by ultra-filtration; also that the component which diffuses in dialysis and forms the filtrate in ultra-filtration is a thermo-stable, inorganic activator. Raper has partially confirmed Haehn's finding, but only in the juice of fresh potatoes; he also believes that this activator is organic.

It has been frequently suggested that traces of iron or manganese are essential for the activity of oxidases. This is suggested by Fenton's reaction. As remarked by Bayliss, the investigations of Willstätter and Pollinger however suggest caution, for they found that the activity of peroxidase preparations was not in proportion to the iron-content. Since electrodialysis will remove all inorganic impurities, the role of manganese and iron in the inorganic form can be easily decided.

The crude meal-extract after filtration was dialysed for three days against running distilled water to remove most of the protein and then one portion was put in the electro-dialyser and another submitted to ordinary dialysis. The electro-dialysis was at 30 volts. By having the anode space thrice as large as the cathode space and allowing a very low current to pass, acidification was avoided. Soon after the electro-dialysis was finished, the solutions were compared for their activities on tyrosine. Two sets of experiments have been conducted, one with parchment and another with collodion membranes.

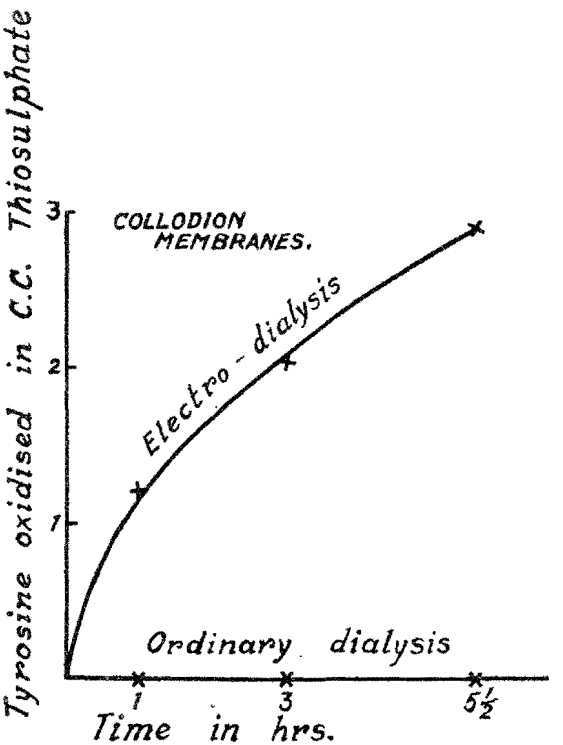

Tyrosine oxidised in C.C. Thiosulphate
3
2
1
COLLODION MEMBRANES.
Electro - dialysis
Ordinary dialysis
1
3
5½
Time in hrs.

Fig.X

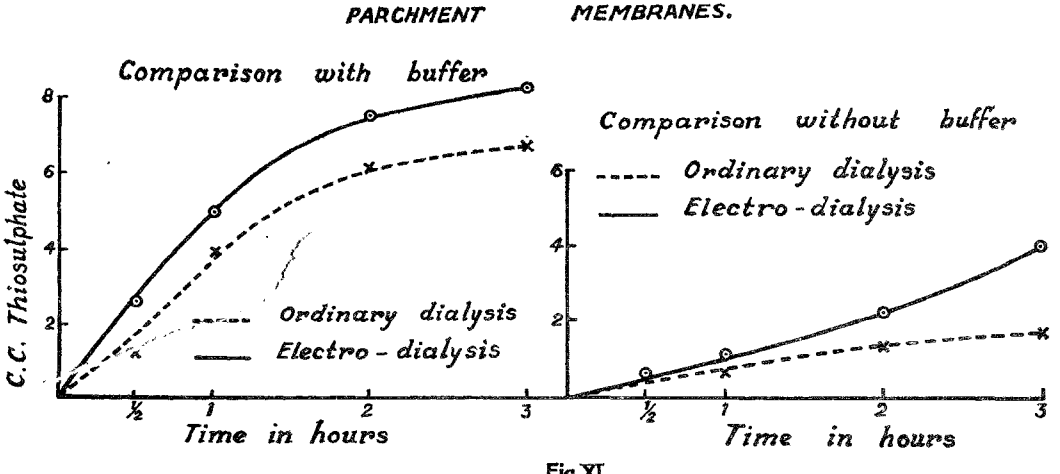

PARCHMENT MEMBRANES.
Comparison with buffer
C.C. Thiosulphate
8
6
4
2
Ordinary dialysis
Electro - dialysis
½
1
2
3
Time in hours
Comparison without buffer
Ordinary dialysis
Electro - dialysis
6
4
2
½
1
2
3
Time in hours

Fig.XI

In both these cases the electrodialysed enzyme was more active, with buffer or without buffer. These experiments indicate that inorganic salts do not play any great part in the action of this enzyme and that if an activator is present it must be in the colloidal condition, or at least bound to a colloidal surface (c. f. Onslow, *Biochem. J.*, 1928, **22**, 1327). The results are given in Tables XIII and XIII A and Figures X and XI.

TABLE XIII (Collodion membrane).

The reaction mixture contained 50 c. c. tyrosine (0·05 per cent.). 50 c. c. enzyme solution and 10 c. c. toluene. The temperature was 34·6°.

Time in hours	Tyrosine oxidised in c. c. thiosulphate	
	Electro-dialysis	Ordinary dialysis
1	1·2	..
3	2·0	...
5·5	2·8	Light rose coloration

TABLE XIII A.

(Parchment membranes).

Composition of the reaction mixture.

No.	Method of preparation	Tyrosine 0·1 per cent.	Buffer P_H 6·52	Water	Toluene	Enzyme solution
1	Electro-dialysis	50 c. c.	10 c. c.	20 c. c.	10 c. c.	20 c. c.
2	Ordinary dialysis	50 c. c.	10 c. c.	20 c. c.	10 c. c.	20 c. c.
3	Electro-dialysis	50 c. c.	...	30 c. c.	10 c. c.	20 c. c
4	Ordinary dialysis	50 c. c.	...	30 c. c.	10 c. c,	20 c. c

Temperature of the reaction 25°.

Time in minutes	Tyrosine oxidised in c. c. thiosulphate			
	With buffer		Without buffer	
	Electro-dialysis	Ordinary dialysis	Electro-dialysis	Ordinary dialysis
30	2·6	1·2	0·6	0·3
60	5·0	3·9	1·1	0 6
120	7·5	6·1	2·2	1·3
180	8·2	6·6	4·0	1·6

Ultra-filtration.—These experiments were conducted to seek evidence for the assumption that tyrosinase is a mixture of components and also to test Haehn's statements (*Biochem. Z.*, 1920, **105**, 159) that the ultra-filtrate is necessary for the action of the enzyme. Experiments were made with an improved form of apparatus described by Brukner (*Z. Ver. Deut. Zucker Ind.*, Band **76**, *Technischer Teil*, vol. 837, June 1926; Dissertation, University of Gottingen) using Zsigmondy's ultra-fine filters as well as parchment. Compressed air was used for pressure and the experiments were made without stirring. Filtrates collected up to a pressure of 50 atmospheres were found to have no activity; on the filter there was much precipitate which on dispersion in water was found to be active. The residual liquid on the filter was slightly more active than the untreated solution; addition of ultra-filtrate did not cause any increase in activity. These experiments clearly show that the tyrosinase from *Dolichos lablab* does not lose activity on ultra-filtration: they also indicate the probable absence of any activator and that if present it must be colloidal or bound to a colloidal carrier; further that the enzyme is of a coarse dispersity. The possibility of obtaining the solid enzyme free from all crystalloidal impurities is also clearly indicated. About 150 c.c. of the dialysed enzyme solution were put in the ultra-filtration apparatus and the filtrates collected at pressures of 25 and 50 atmospheres. The results are given in Table XIV.

TABLE XIV.

No. of filtrate	Pressure at which collected	Volume in c.c.
1	25 atm.	28
2	50 atm.	28
3	50 atm.	52
Residual liquid on the filter ...	... ...	38
Total ...	... ...	146

The sticky black precipitate on the filter was dispersed in 20 c.c. of distilled water and the activities of the several fractions and the dispersed solid compared with that of the untreated solution. The next day the residual liquid (preserved in the ice-box) was found to have deposited a large amount of solid; the clear upper layer of liquid was however still very active.

TABLE XIV A.

Fraction collected at	Activity in c. c. thiosulphate
(Untreated)	7·5
25 atm.	0
50 atm.	0
50 atm. (2nd fraction)	0
Residual liquid	7·6
The dispersed precipitate	7·7

The concentration of tyrosine was 0·05 per cent. and the reaction was carried out at P_H 6.52 and temperature 30°.

Ultra-filtration with parchment membranes.—The experimental details were the same as with Zsigmondy's ultra-fine filter. About 135 c.c. of the enzyme solution were used. The results are given in Tables XV and XV A.

TABLE XV.

No. of filtrate	Pressure at which collected	Volume in c. c.
1	25 atm.	21
2	50 atm.	25
3	50 atm.	76
4 Residual liquid on the filter ...	... ...	7
Total ...	... ...	129

The precipitate on the filter was dispersed in 25 c.c. of water and the activities of the various fractions compared with a sample of the untreated solution.

TABLE XV A.

Fraction collected at	Activity in c. c. thiosulphate
(Untreated)	7·6
25 atm.	0
50 atm.	0
50 atm.	0
Residual liquid	5·3
The dispersed precipitate	10·0

Effect of adding the Ultra-filtrate to the residual liquid.

TABLE XVI.

—	Residual liquid from Zsigmondy filter (5 c.c.) + 5 c.c. water.	Residual liquid from Zsigmondy filter (5 c.c.) + 5 c.c. ultra-filtrate.
Activity in c.c. thiosulphate ...	9·3	9·2

SUMMARY.

Active preparations of tyrosinase stable in aqueous solution for a considerable length of time have been obtained from the ungerminated seed of *Dolichos lablab*.

The solubility relations of the enzyme indicate its colloidal nature.

The optimum P_H and the isoelectric point of the enzyme are in the neighbourhood of 6·5.

The kinetics of tyrosinase action under varying conditions of substrate concentration, enzyme concentration, hydrogen-ion concentration and temperature have been investigated. That the reaction can proceed to completion is also indicated.

The enzyme in aqueous solution is stable up to 70°.

Tyrosinase is activated by exposure to the rays from a quartz mercury arc.

The influence of hydrogen peroxide in the action of the enzyme has been studied.

Preparations practically free from electrolytes have been obtained for the first time by subjecting tyrosinase to electro-dialysis, by which tyrosinase is considerably increased in activity.

Evidence based on electro-dialysis and ultra-filtration experiments shows that contrary to Haehn's view inorganic salts are not necessary for the action of this enzyme, and that, if any co-enzyme is present, it must be colloidal, or associated with a colloidal carrier.

We desire to express our indebtedness to Prof. R. V. Norris for the keen interest he has taken in this work.

Department of Bio-Chemistry,
Indian Institute of Science,
Bangalore.

[Accepted, 25–4–29.]