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ACTIVATION OF *VICIA FABA* (L.) TYROSINASE AS EFFECTED BY DENATURING AGENTS

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Abstract—The heterogeneity of *Vicia faba* preparations is shown to be unrelated to the effect of denaturing agents. Conditions for maximal promotion of the latent tyrosinase activity have been studied and anionic detergents are shown to be better activating agents than urea and acid treatments. It is suggested that the activation process involves a limited conformational change such as a swelling of the protein rather than a dissociation or aggregation. In high concentrations of urea the cresolase activity is less easily inhibited than catecholase. The urea activated enzyme had a somewhat lower affinity for *p*-cresol than the manoxal activated enzyme. Guanidine salts are also activating agents but frequently inhibition of the activated form is encountered. Guanidine hydrochloride produces a stronger inhibition than sodium fluoride.

INTRODUCTION

THE tyrosinase isolated from leaves of *Vicia faba* (L.) has been described by Kenten as a latent enzyme. He showed that the activity of crude extracts of broad bean can be enhanced from twenty to fifty times by treatments with such activators as acid, alkali,[1] anionic detergent[2] and pepsin.[3] More recently urea[4] and macromolecules, such as polygalacturonic

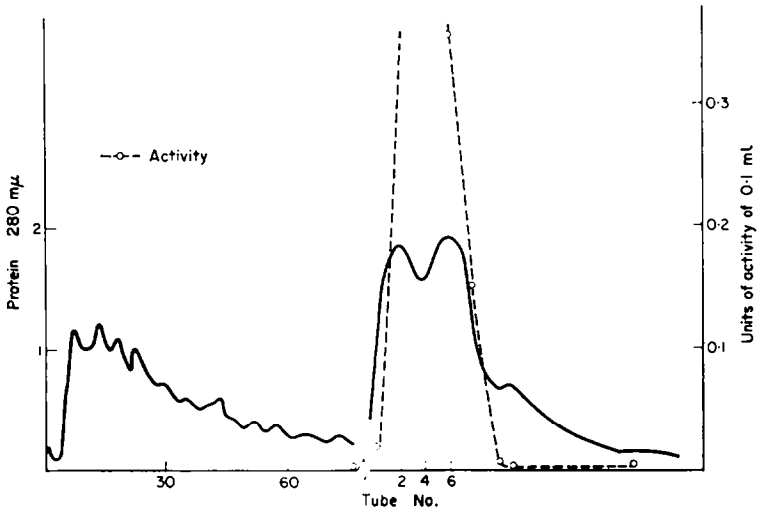

FIG. 1. ELUTION DIAGRAM OBTAINED BY CONTINUOUS ELECTROPHORESIS. (See text for details.)

[1] R. H. KENTEN, *Biochem. J.* **67**, 300 (1957).
[2] R. H. KENTEN, *Biochem. J.* **68**, 224 (1958).
[3] R. H. KENTEN, *Biochem. J.* **65**, (1955).
[4] D. A. ROBB, *Nature* **207**, 503 (1964).

acid,[5] have also been described as activating agents. Consideration of these agents indicates that the activity of this enzyme is promoted by mildly denaturing conditions—circumstances which normally cause a reduction in the activity of many enzymes. A study of the process of denaturation forms the basis of the present paper.

TABLE 1. THE EFFECT OF ACTIVATING SYSTEMS ON VARIOUS TYROSINASE PREPARATIONS

Activating* system	Enzyme† fraction	Maximum activity released compared to manoxal (%)
Manoxal	Initial extract	100
6 M Urea		50
Acid		33
Acid + manoxal		75
Acid + 7 M urea		12
Manoxal + 7 M urea		50
7 M Urea + manoxal		50
Manoxal	Ammonium sulphate	100
6 M Urea		66
Acid		20
Manoxal	*Fraction* 2	100
Acid		20
Manoxal	*Fraction* 4	100
Acid		20
2·8 – 5·6 M Urea		50–60
Acid + 4·2–5·6 M urea		45
Manoxal + 5·6 M urea		50
Manoxal	*Fraction* 6	100
Acid		50
2·8–4·2 M urea		50
Acid + manoxal		80
Acid + 1·4 M urea		35
Manoxal + 2·8 M urea		83
(Manoxal + 5·6 M urea)		(36)

* Activating conditions: Acid—0·5 ml 0·2 M tris-HCl, pH 2·8 was incubated with 0·1–0·3 ml enzyme (final pH 3·0) for various times and diluted to 3·4 ml (with tris-buffer) for assay containing homocatechol (4 mg) to give a pH 5·0. Urea—enzyme assayed in various concentrations of urea in 0·1 M phosphate–0·05 M citrate buffered at pH 4·7. Manoxal OT—routine assay, pH 5.0. Two activators used—enzyme incubated with first one using optimal conditions (e.g. 0·2 ml enzyme + 0·5 ml concentrated urea sufficient to produce optimal molarity) and then exposed to second and assayed under optimal conditions for the second activator.

† The specific activities of the fractions are: initial extract 5·0; ammonium sulphate 8·7; zone electrophoresis 60·0. Fraction 2 and 6 are equivalent to the β and α fractions described previously[6] and isolated as the two protein peaks by electrophoresis on cellulose (Fig. 1): fraction 4 is an intermediate consisting of a mixture of the two.

The existence of multiple forms of tyrosinase in *Vicia faba* and methods for their purification and partial separation of the iso-enzymes has already been described.[6] Incomplete

[5] B. J. DEVERALL, *Nature* **189**, 311 (1961).
[6] D. A. ROBB, L. W. MAPSON and T. SWAIN, *Phytochem.* **4**, 731 (1965).

separation renders characterization of the iso-enzymes difficult but an attempt has been made to determine whether the forms can be distinguished by their reactions with denaturing agents. Two main fractions of broad bean tyrosinase can be isolated and each shows a large degree of activity when assayed directly with either mono- or orthodihydricphenols. In contrast to crude extracts, treatments with urea and acid do not promote the tyrosinase activity, but both fractions show increased activity ($\sim$ twofold) when assayed in the presence of bis (2-ethylhexyl)sodium sulphosuccinate (manoxal OT).[6] One possible explanation for the different behaviour of crude and highly purified tyrosinase fractions is that during the purification, especially at stages involving absorption and desorption on columns of triethylaminoethyl-cellulose and hydroxyl-apatite, sufficient denaturation occurs to activate the enzyme *per se*. Such denaturation might differ for each iso-enzyme and thereby any distinguishing feature may be destroyed or augmented during purification. Therefore the experiments described below were carried out with an enzyme which had been subjected to as few purification steps as necessary to resolve the iso-enzymes. After extraction from an acetone powder and fractionation with ammonium sulphate, the enzyme was subjected to electrophoresis on cellulose. The elution diagram for this latter step (Fig. 1) shows that the activity is associated with two partially resolved protein peaks and on analysis by starch gel electrophoresis it was demonstrated that the tubes corresponding to the peaks contained tyrosinase giving two distinct multi-banded patterns similar to those obtained on more extensive purification.[6] The efficiency with which treatment with acid, urea and manoxal OT enhanced the tyrosinase activity of these fractions and also of aliquots obtained at each stage of the purification procedure, was tested using 4-methylcatechol as the substrate. The results (Table 1), show that all the samples examined were activated most efficiently by incubation with manoxal. Urea and acid employed either alone or in succession were at best only 80 per cent as effective. In this and all such experiments which we have conducted it has proved impossible to distinguish unequivocally the observed heterogeneity of tyrosinase by treatment with denaturing agents, and thus the existence of the various forms of tyrosinase is disregarded in this study.

CONDITIONS FOR OPTIMAL ACTIVATION

The results presented in this section were obtained using homocatechol as substrate; similar observations were found with *p*-cresol.

Activation by Acid and Alkali

The results of Kenten[1] that exposure for a limited time to pH of 3·0–3·5 or 10–11·5 leads to activation, have been confirmed. The period of incubation at extreme pH leading to maximum activation is dependent on the nature and concentration of the proton donor or acceptor employed and also on the purity of the enzyme preparation; it is also critical since the activated enzyme is destroyed in these conditions. Solutions of low ionic strength favour activation and greater efficiency was obtained when buffers other than acetate and phthalate were employed. An optimal effect was obtained by incubation in a tris–HCl buffer pH 3·0 with ionic strength 0·1, for a defined period between 1 and 3 min.

Activation by Anionic Detergents

Activation by manoxal OT or by sodium dodecyl sulphate (SDS) is preferable to pH treatments since the activated enzyme is more stable in their presence. However, prolonged

contact with excess detergent—especially with SDS—does result in large losses of activity. At the optimum pH (5·1), activation by both SDS and manoxal OT was found to be rapid, and for the oxygen cell assay it was determined that 8 μmole in a total volume of 3·1 ml brought about complete activation in 1 min. A linear relation between increase in activity and concentration of SDS employed (see Fig. 2) is obtained only after a definite concentration of SDS is attained. This concentration threshold is $\sim$60–120 μM.

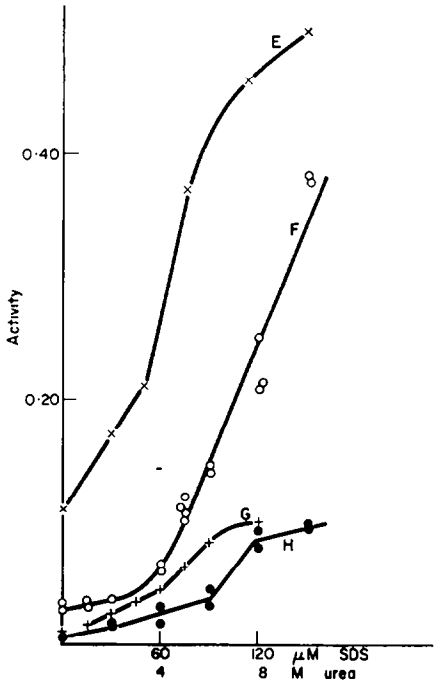

FIG. 2. THE RELATION BETWEEN THE INITIAL RATE OF DISAPPEARANCE OF OXYGEN AND CONCENTRATION OF ACTIVATING AGENT.

Curve E relates to urea, curves F, G and H, to SDS. All measurements were made at pH 5·0 with the oxygen cell in either a total volume of 3·4 ml (Curves E, F and G) or 6·8 ml (Curve H). Curve F and Curves G and H relate to systems containing 0·40 and 0·12 enzyme units respectively.

Activation by Polyanions

Deverall[5] has demonstrated that polysaccharides such as polygalacturonic acid and carboxymethylcellulose activate most effectively in the pH range 4·0–4·5. The efficiency of carboxymethylcellulose is dependent on chain length and the degree of substitution with the carboxymethyl group. When similar grades of carboxymethyl- and methyl-celluloses were compared at a concentration of 0·15 per cent the former were found to be three times more effective.

Activation by Urea and Guanidine

Urea and guanidine salts in high concentrations were shown to activate optimally at pH 4·5–4·7. Below this pH range activity was lost irreversibly. Studies with guanidine salts are complicated by the fact that anions, particularly monovalent anions of weak acids, are inhibitors of the activated enzyme. Thus guanidine phosphate and nitrate are better

activators than the halide salts because at pH 5 they produce less inhibition of the active enzyme. Guanidine acetate besides being strongly inhibitory at pH 5 also activates to only a small extent (20 per cent or less as effective as guanidine phosphate). Formamide and ammonium sulphate in high concentration also have an activating effect, albeit a minor one. Like urea both these agents activate optimally at pH 4·5–4·7.

The effect of various urea concentrations on the activity of equal amounts of enzyme at pH 5·0 is shown in Fig. 2. The addition of increasing amounts of urea initially gives a proportional increase in activation up to about 4 M: after this the activation produced by each increment of urea is much greater up to the maximum. This process can be followed by starch gel electrophoresis. Figure 3 shows that after incubation in 3 M urea at pH 5·0 the starch gel distribution of tyrosinase was not significantly altered; with 6 M urea, however, activation is associated with the appearance of a new electrophoretic component of higher mobility. Samples which had been either incubated in a 6 M urea at neutral pH—a condition

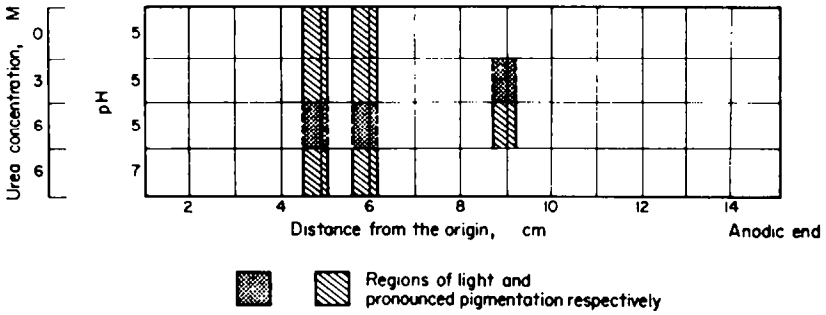

FIG. 3. DIAGRAMMATIC REPRESENTATION AFTER ELECTROPHORESIS ON AN 11% STARCH GEL AT 8 V/cm FOR 15 hr, OF THE EFFECT OF PRIOR INCUBATION IN UREA ON THE DISTRIBUTION OF ENZYME ACTIVITY.

Tyrosinase was located by immersing the gel in dehydroxyphenylalanine solution (1 mg/ml) when regions of activity became pigmented with melanin.

causing little activation—or activated at lower pH and then dialysed to remove the urea, did not contain this new component.

Activation by Dilution

When the activities of aliquots of one enzyme preparation which have been stored at different dilutions, are compared at equivalent concentrations with the addition of an activating agent, the one stored at highest dilution is found to be most active. Furthermore, when an activator is omitted from the assay system the response between activity and the enzyme concentration is linear only at the lowest enzyme concentrations where the rate observed is equivalent to that obtained in the presence of manoxal OT—i.e. in this region the enzyme is activated.[6] Thus dilution, unlike other physico-chemical treatments such as heating or freezing and thawing, does enhance the activity of the enzyme. The converse of this observation, that dilution causes a loss in specific enzyme activity has been reported for several enzymes including β-glucosidase and hyaluronidase.[7]

[7] P. BEMFIELD, H. C. BERNFIELD, J. S. NISSELBAUM and W. H. FISHMAN, *J. Am. Chem. Soc.* **76**, 4872 (1954); P. BEMFIELD, S. JACOBSON and H. C. BERNFIELD, *Arch. Biochem. Biophys.* **69**, 198 (1947); P. BEMFIELD, L. P. TUTTLE and R. W. HUBBARD, *Arch. Biochem. Biophys.* **92**, 232 (1961).

The Effect of pH

The effect of pH on the activation process and also on the catecholase activity of the treated enzyme is illustrated for the acid, urea and manoxal OT treatments in Figs. 4-6.

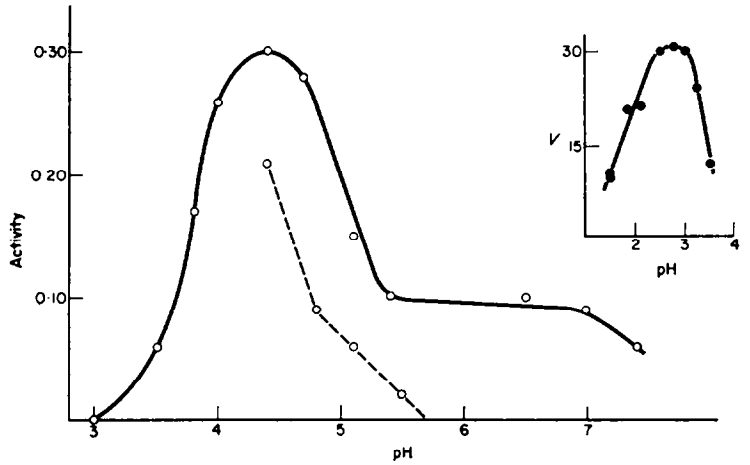

FIG. 4. pH-ACTIVITY CURVE FOR AN ACID-ACTIVATED ENZYME.

The dashed line relates to the activity of the untreated preparation. Inset activity produced by the addition of the enzyme to solutions of 0·003 M hydrochloric acid adjusted to various pHs with tris, and then after 3 min readjusting the pH to 5·0 and immediately determining the activity.

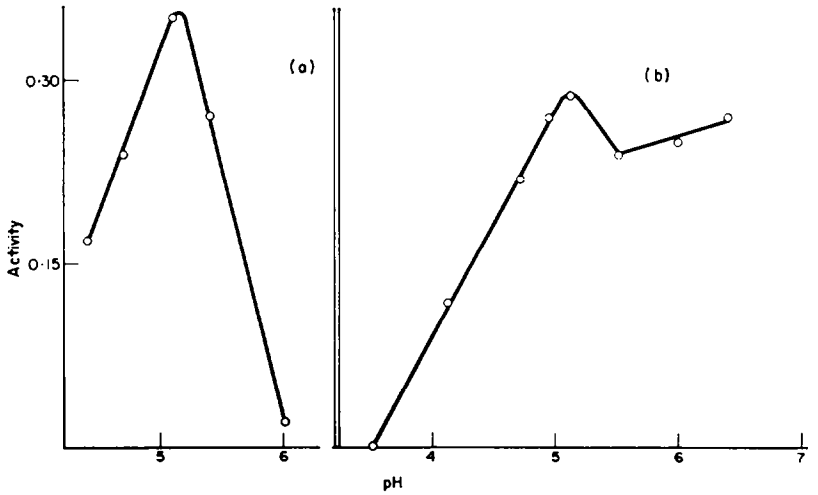

FIG. 5. (a) THE EFFECT OF A 2 MIN INCUBATION WITH 190 μM SDS AT VARIOUS pH VALUES ON THE ACTIVITY OF TYROSINASE DETERMINED AT THE pH OF INCUBATION; (b) pH-ACTIVITY CURVE FOR A PREPARATION ACTIVATED BY STORAGE FOR 5 MIN IN 13 mM MANOXAL AT pH 5·0.

The general form of the pH activity curve is similar in all three cases, showing an optimum at either pH 4·5–4·7 or pH 5·1 and a shoulder of virtually unchanged activity in the pH range 6·0–7·5. However, the magnitude of the shoulder compared to the optimum varies from a tenfold reduction with urea to only about 80 per cent reduction with manoxal.

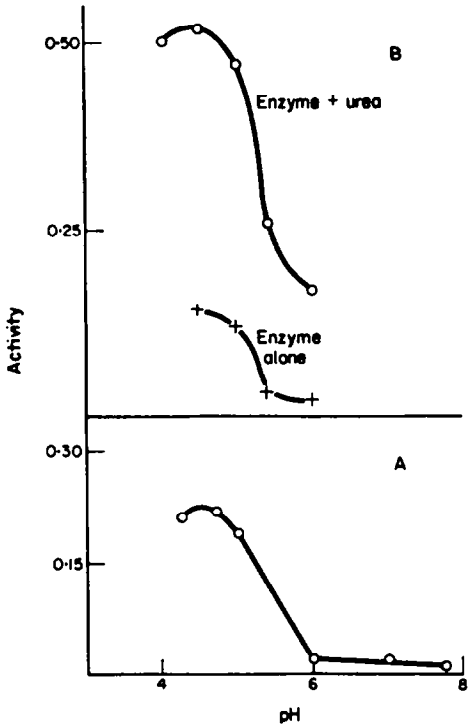

FIG. 6. (a) pH-ACTIVITY CURVE OF TYROSINASE AFTER INCUBATION IN 10 M UREA AT pH 4·9 FOR 15 MIN, THE CONCENTRATION OF UREA IN THE ASSAY MEDIUM IS 0·05 M; (b) THE ACTIVITY OF THE TYROSINASE PREPARATION AT SEVERAL pH VALUES DETERMINED IN THE PRESENCE AND ABSENCE OF 10 M UREA.

Effect of Temperature

In order to ascertain whether heating enhanced the activity of a preparation which was activated by both manoxal and urea, samples were examined after incubation for 1–45 min. at temperatures between 45–70°. The pH of the incubation medium was varied between 4·5–8·0 using acetate, phosphate and tris-hydrochloride buffers at three molarities (0·05, 0·1 and 0·2 M respectively) and the results of assays made at pH 5. The results showed that no activation occurred. For example, when a preparation which could be activated tenfold by prior treatment with manoxal, was heated at 69·5° in 0·2 M phosphate, pH 6·8 for 10 min the enzyme was totally inactive unless a denaturing agent (such as manoxal) was also present. The activity which was recovered by manoxal OT amounted to 25 per cent of that initially present. Thus the results indicated that both the active and inactive forms of the enzyme are labile to heat treatment and it appears that the inactive form is converted directly to a denatured state. A similar phenomenon is observed when the enzyme is incubated in urea at pH 10·5.

Agents Without Effect on the Inactive Enzyme

Certain chemicals which have been regarded as denaturing agents, such as lithium bromide,[8] dioxane and ethylene glycol,[9] neither enhanced the activity of the latent enzyme nor

[8] J. BELLO and H. R. BELLO, *Nature* **194**, 681 (1962).
[9] E. FREDERICQ, *Ciba Found. Colloq. Endocrinol.* **9**, 89; W. KAUZMANN, *Advan. Protein Chem.* **14**, 1 (1959).

prevented the activation caused by manoxal OT. Kenten[2] has shown that cationic and nonionic detergents are also ineffective.

THE REVERSAL OF ACTIVATION

In some instances it has proved possible to demonstrate the reversal of activation which accompanies the removal of the activating agent. Most investigations on this subject have centred on acid activation for not only is the removal of the activating species readily achieved (by neutralization) but also the total recovery of enzyme is higher than that obtained after removal of the other activating agents. When a crude preparation is activated by exposure to pH 3 and then stored at pH 7, the activity it shows by analysis at pH 5 decreases with time, often disappearing almost completely after 10 hr storage at 5°. However by repetition of acid treatment or addition of manoxal the activity is rapidly regained thereby indicating that the enzyme can revert from an active to an inactive form. Furthermore, it is possible to preserve the activity released by acid treatment after neutralization, by adding a reagent which performs normally as a weak activator such as ammonium sulphate.

Adjustment of pH is insufficient to cause a reversal to the latent form with enzymes activated by excess urea, manoxal, or sodium dodecyl sulphate or carboxymethyl-cellulose. However, the enzyme recovered after dialysis against a neutral buffer is in a predominantly latent form when urea, ammonium sulphate or carboxymethyl-cellulose are employed. The conversion to the latent form is low, ~30 per cent, but better than the zero yield obtained when attempts are made to remove by dialysis excess manoxal OT or sodium dodecyl sulphate. Nevertheless, a reversal of manoxal OT activation has been demonstrated. When a fully activated preparation is subjected to gel filtration on a column of "Sephadex" G-75 20 per cent of the activity applied is recovered. Manoxal OT, but not urea or acid, enhances the activity of this enzyme twofold, thereby demonstrating that a reversion to a partially latent state has occurred.

THE MODE OF ACTIVATION

One explanation for the latency of broad bean tyrosinase is that the prosthetic group of the inactive form of the enzyme is unable to combine with either oxygen or the phenolic substrate or both. Copper is known to be essential for the activity of tyrosinase, and thus it seemed appropriate to determine whether the copper-chelating agents, cyanide and diethyldithiocarbamate (DIECA), which are of a similar order of size to the substrate molecules, would combine equally well to both the inactive and activated forms of the enzyme.

Accordingly enzyme samples were activated with either acid or manoxal OT and the chelating agent was added. After 15 min the samples were dialysed for 24 hr against two changes of buffer under conditions which maintained the enzyme in an active condition, and then they were analysed for activity, before and after the addition of manoxal OT. An unactivated sample and controls from which the complexing agent was omitted were also subjected to the same procedure. A set of results obtained with DIECA is given in Table 2 together with a summary of similar experiments. The results show that in the inactive form the copper is either less accessible to, or alternatively has a lower affinity for, the complexing agents. Hence, it can be concluded that activation is accompanied by an alteration in the environment of the prosthetic group so that it combines more readily with complexing agents.

The process was further investigated using the ultracentrifuge.* The sedimentation coefficients of acid and manoxal OT activated enzymes were computed (by the sedimentation velocity method) and compared with the untreated enzyme. The untreated enzyme preparation purified 100-fold, and dissolved in a solution of 0·012 M disodium phosphate, 0·115 M potassium dihydrogen phosphate, 0·05 M sodium chloride, pH = 6·7 and ionic strength of 0·1, sedimented one peak with a sedimentation coefficient S_{20} of 4·3 *S*. The activity of this enzyme was enhanced twelvefold by addition of manoxal (final concentration 0·3%) and sixfold by exposure to pH 3 for 2 min followed by neutralization. The sedimentation patterns of these two samples, determined immediately after activation and in the same buffer, were similar to the untreated specimen yielding S_{20} values of 4·4 *S* and 4·0 *S* for the manoxal and acid treatments respectively. It is to be noted that any change in viscosity produced by the presence of the activating agents in the analysis has been neglected.

TABLE 2. THE EFFECT OF COPPER CHELATING AGENTS ON LATENT AND ACTIVE TYROSINASE

Inhibitor concentration (mM)	Activating agent	Percentage inhibition* of unactivated enzyme	Percentage inhibition* of activated enzyme
Dieca 1	Acid†	37	75
0·33	Acid†	17	80
KCN 3·3	Manoxal OT‡	37	57
3·3	Manoxal OT	33	65
1·7	Manoxal OT	25	54

* Determined using the oxygen cell assay with homocatechol as substrate.

† Acid activated and stored in 2 M ammonium sulphate, pH 6, which was also used for dialysis.

‡ After incubation with 1 mM manoxal the samples were dialysed against phosphate pH 6·0 containing 1 mM manoxal.

It is inferred from the similarity of the sedimentation data, especially for activation by manoxal OT, that the increase in activity is accompanied neither by aggregation nor by the liberation of a masking protein of substantial molecular weight. However, the data is compatible with mechanisms postulating that activation is accomplished either by the liberation of a low molecular weight unit, such as a peptide or merely of rearrangement of the tertiary structure. However, since prior dialysis of an acid treated enzyme at a neutral pH in the presence of ammonium sulphate, gives an almost complete reversion (70–80 per cent) to an inactive form when the ammonium sulphate is dialysed away, the conjecture that a dialysable peptide fragment is important in the activation or inactivation process, can be excluded. The experiment reported in the previous section where gel filtration on Sephadex G-75 resulted in a partial reversion is also in accord with this conclusion. Inspection of Fig. 7 shows that mild denaturing conditions lead to the production of active enzyme while stronger denaturing conditions cause irrecoverable losses in enzyme activity. Thus the activation process can be represented equally well by a system which regards the active enzyme as a transitory state (e.g. Inactive Enzyme⇌Active enzyme→Denatured state, devoid of activity).

* We are indebted to Dr. P. Johnson for his co-operation in these analyses.

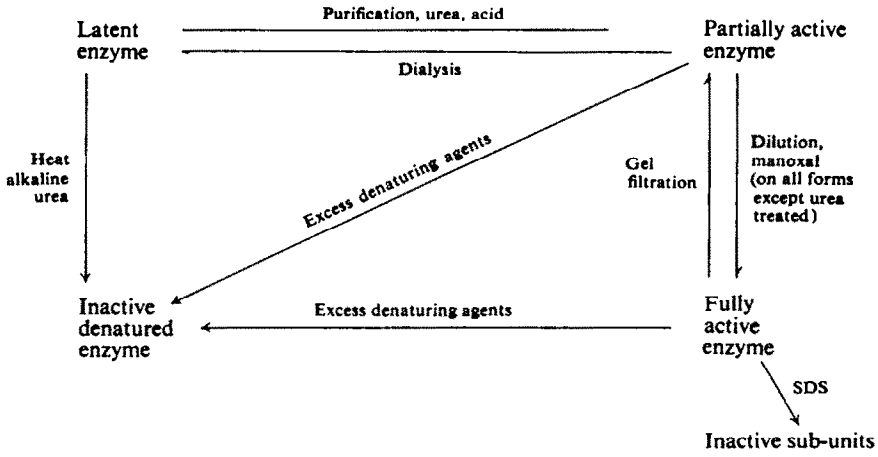

FIG. 7. A SCHEME SUMMARIZING THE EFFECTS OF DENATURING CONDITIONS ON THE ACTIVITY OF *Vicia* TYROSINASE.

Milder denaturing conditions (dilution, incubation for a short time at low pH or in dilute detergent solutions) affect mainly the equilibrium concentrations of inactive enzyme and active enzyme by favouring the formation of the latter. Stronger denaturing conditions, incubation at a lower pH or in more concentrated detergent solutions ($> 10^{-1}$ M) or urea solutions (2–7 M) which are normally used to show activation, also promote some formation of irreversibly denatured material and are thus less efficient activators. Such a scheme explains why detergents fail to increase the activity of urea treated samples. Furthermore if the reasonable assumption is made that the irreversibly denatured material cannot combine with substrate, guanidine would be expected to inhibit the active enzyme competitively. The effect of more drastic denaturing conditions has been further investigated for only one condition. An ultracentrifugal analysis of an enzyme preparation, whose activity had been destroyed by storage at pH 5·0 for 2 hr in 1 % sodium dodecylsulphate showed that the component with a sedimentation coefficient, $S_{20}=4{\cdot}3$ had disappeared completely and been replaced by a species with a much lower sedimentation coefficient (the value uncorrected for temperature or viscosity approximates to 1·6 *S*).

Kinetic Studies on the Effect of Urea and Guanidine Hydrochloride on the Activated Enzyme

One is handicapped in this investigation because the activated state is transitory. On the one hand it is difficult to prevent reversion to the latent state and yet on the other to prevent irreversible loss of activity, and tyrosinase maintained in manoxal OT or urea gradually loses its activity. Work on this aspect has therefore been confined to a comparison of kinetic measurements made in the presence of the activators. Hence in a manometric assay the enzyme was added from the sidearm of the flask to a mixture of buffer, substrate and urea or manoxal OT, and in oxygen cell measurements the enzyme was added to urea or manoxal and after 10 min the substrate was added. In order to remove the lag period which proceeds oxidation of *p*-cresol when measured by the manometric assay and thus to make the conditions more alike for the oxidation of mono- and diphenols, a trace of homocatechol was added to the solution in the main compartment.

Observations made using both assays showed that in urea, activity toward homocatechol

was more easily inhibited than activity toward *p*-cresol. This is illustrated by the results quoted in Table 3 where a fraction, purified two thousand times, was used.

The progress curves showed that in common with other tyrosinases[10] reaction inactivation occurred during the oxidation of homocatechol, and the normal linear rate for *p*-cresol oxidation was given only in urea concentrations less than 3 M. These results indicate that a distinction can be made between the two activities of broad bean tyrosinase—that by measuring the homocatecholase:cresolase ratio in high concentrations of urea an abnormally low value is obtained. The kinetics of *p*-cresol oxidation in 2 M urea and manoxal OT were compared using the manometric method. In both cases the relation between rate of oxygen uptake and *p*-cresol concentration was typical of an enzyme catalysed reaction, and the Michaelis constants for the urea and manoxal OT activated systems were determined to be 5–7 mM and 1–3 mM respectively. Chromatographic analysis,[11] of the products formed

TABLE 3. THE EFFECT OF VARYING CONCENTRATIONS OF UREA ON ENZYMIC ACTIVITY TOWARD MONO- AND *O*-DIHYDRIC-PHENOLS

Concentration of urea (M)	Activity (ml/min) O_2 toward	
	p-cresol	homocatechol
0	4·9	3·5
1	6·6	3·5
2	6·6	3·5
3	6·0	3·2
4	4·2	2·0
5	3·4	0·9

The manometric assay was used, activity being determined at pH 4·7 in 3 ml containing EDTA (6 μM), 0·1 M phosphate, 0·5 M citrate, various concentrations of urea adjusted with citric acid to a pH of 4·7 and 4 mg of either homocatechol or *p*-cresol: for assay of "Cresolase" 0·01 mg of homocatechol and a tenfold greater concentration of enzyme were present.

in the presence of excess ascorbic acid (0·04 M) by the urea activated system demonstrated that 4-methylcatechol was the sole diphenol produced. Thus it appears that urea does not drastically alter the normal enzymic reaction.

The activities of broad bean tyrosinase toward *p*-cresol and homocatechol also show somewhat different behaviour with guanidine hydrochloride. In this experiment the inhibition produced by the guanidine salt with an enzyme fully activated by manoxal OT was investigated. The inhibition with guanidine hydrochloride followed initially a similar course for both substrates being gradual at first before reaching a range of concentration where inhibition was constant. With *p*-cresol as substrate, however, a third phase was found where a further rise in the concentration of guanidine hydrochloride again produced a rise in inhibition (Fig. 8). In the range where inhibition is competitive the value of dissociation constant of the enzyme–guanidine complex with homocatechol as a substrate is about 0·002 M. This value may be compared to that for the dissociation constant of the sodium fluoride enzyme

[10] J. H. NELSON and C. R. DAWSON, *Advan. Enzymol.* **4**, 99 (1944).
[11] E. C. BATE-SMITH, *Chem. & Ind. (London)* 1457 (1954).

complex where K_I is 13 mM, and also to the results of Rajagopalan *et al.*[12] who reported that mushroom tyrosinase (which is isolated in a fully active form) is competitively inhibited by urea, guanidine and formamide (K_I values 1·7, 0·25 and 1·8 M respectively) and non-competitively by sodium dodecylsulphate when catechol is used as a substrate.

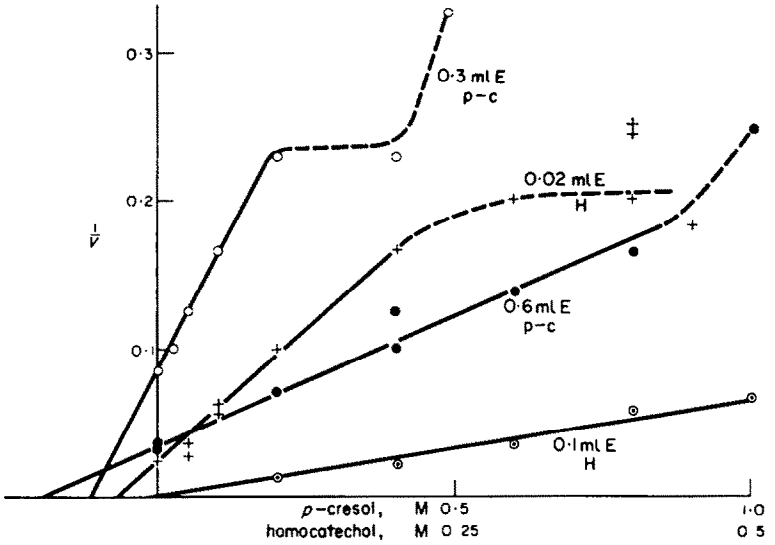

FIG. 8. THE PLOT OF THE RECIPROCAL OF THE ENZYME VELOCITY AS MEASURED WITH THE OXYGEN CELL AND THE CONCENTRATION OF GUANIDINE HYDROCHLORIDE AT VARIOUS ENZYME CONCENTRATIONS BUT CONSTANT SUBSTRATE CONCENTRATIONS (4 mg OF EITHER 4-METHYLCATECHOL (H) OR *p*-CRESOL (p-c) IN 3·4 ml).

DISCUSSION

Masked forms of tyrosinase and *o*-diphenol oxidase have been isolated from organisms at various phylogenetic levels.[13–21] Of particular interest are the "protyrosinases" of the Insecta *Melanoplus differentialis*,[17] *Musca vicina* Macquart[18] and *Tenebrio molitor*,[20] the protyrosinase of crayfish serum (*Cambarus* sp.),[20] and the latent *o*-diphenol oxidase of *Caliphora erthyrocephala*.[21] Mild denaturation such as treatment with anionic detergents or incubation at extremes of pH, has been show to cause activation in all these examples except *Caliphora*. In the instances tested (*Caliphora*[21] and *Vicia*[3]) limited proteolytic attack is also effective.

We concurred previously[4] with the interpretations of Bodine *et al.*[22] and Inaba *et al.*[23]

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that a limited conformational change accompanies activation. At present the active *Vicia* tyrosinases can be distinguished from their latent precursors by their increased mobility on starch gel at pH 8 and their increased susceptibility to copper complexing agents. Apart from the sedimentation data for the *Vicia* system physico-chemical measurements of the activation process have not been made but it seems reasonable to visualize the process as being analogous to the swelling of serum albumen at pH 4 [24] or to the slight rearrangement of the thyroglobulin molecule in 3 M urea or 8 mM sodium dodecyl sulphate, which precedes a major conformational change induced in 8 M urea.[25]

The interaction between thyroglobulin and sodium dodecyl sulphate was studied at a pH greater than the isoelectric point when the two species possess the same overall charge. The production of active tyrosinases by anionic detergents occurs optimally at, or on the alkaline side of the isoelectric point also, and Heyneman and Vercauteren[26] suggested that in *Tenebrio* the activation is mediated through the formation of an enzyme-detergent micelle complex.

Formation of such a complex could conceivably result from electrostatic interaction between the negatively changed micelle and some site on the protein of opposite charge. This hypothesis is attractive for the polyanion, carboxymethylcellulose, which may be regarded as structurally similar to a micelle would be expected to behave similarly. Also Kenten's observations[2] that higher homologues of the paraffin chain acids are more effective activating agents could be explained on the basis that such acids show a greater tendency to form micelles. However the amounts of sodium dodecyl and tetradecyl sulphates which produce extensive activation of the *Vicia* and *Melanoplus*[19] systems are in the range 60–120 μM—less than the critical micelle concentration which can be estimated to be approximately 1 mM for the salt concentrations employed.[27] In addition and in contrast to the *Tenebrio* system[26] both *Vicia* and *Musca*[23] enzymes are unstable in detergent solutions in which large micelles are present. Thus further work is necessary before the idea that the active *Vicia* enzyme is a detergent-protein complex of definite stoichiometry can be excluded.

A major conjecture about the mechanism of action of tyrosinase concerns the possibility that both the hydroxylation of phenols and the hydrogenation of orthodihydric phenols occurs at the same active centre. Early attempts to distinguish the two activities were unsuccessful[28] but more recently such a distinction has been claimed on the basis of Cu^{65} incorporation into the functioning enzyme[29] and also from studies with substrate analogues.[30] However, the fact that the denaturing agent employed to activate the *Melanoplus* protyrosinase also determined the relative ability of the active enzyme to catalyse the two reactions was reported much earlier.[31] That a similar observation can be made with the *Vicia* system should encourage further work on this aspect.

MATERIALS AND METHODS

Chemicals

Manoxal OT 100% was obtained from British Drug Houses. Sodium dodecyl sulphate, purified by recrystallization from ethanol, was a gift from Dr. P. Johnson.

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Imperial Chemical Industries supplied the cetavlon and carboxymethyl- and methylcelluloses in grades B10, B30 and B300. 4-Methylcatechol and *p*-cresol were purified before use whilst all other chemicals were obtained in the highest degree of purity available commercially, and were utilized without purification. Distilled, deionized water was used throughout.

Enzyme Purification

The isolation and purification of *Vicia* tyrosinase have previously been described.[4] Except where otherwise specified the experiments reported in this communication were made with an enzyme preparation which had been extracted from an acetone powder with 0·1 M borate buffer pH 10·0 and subsequently purified by fractional precipitation with ammonium sulphate. By this procedure the enzyme was purified twenty times. This preparation was resolved into two tyrosinase fractions after zone electrophoresis on a cellulose column (23 × 2 cm). This technique was carried out according to Porath and Hjerten[32] but utilizing the continuous elution cell of Naughton and Taylor.[33] A solution of 150 mg of protein in 13 ml was applied to the column and subjected to electrophoresis at 450 V and 15 mA at 5°. The overall yield obtained was 80 per cent with an overall purification of 120-fold. For sedimentation experiments, an enzyme was further purified after ammonium sulphate fractionation by ion exchange chromatography and additional data on urea activation was obtained with an enzyme purified over 2000-fold. The purification of these fractions is reported elsewhere.[6]

Starch Gel Electrophoresis

The method of Smithies[34] as modified by Poulik[35] was used.

Enzyme Assay

Unless otherwise stated assays were performed with an oxygen cell employing homocatechol as substrate (as described previously[6]), or using the manometric method as detailed in the text (cf. [6]).

Unit of Activity

The velocity ordinate of the graphs accompanying this paper is expressed in units relating to the oxygen cell assay. A unit is defined as that amount of enzyme which under the specified conditions of the assay produces an oxygen consumption equivalent to a drop in potential of one mV/min. One of these units was found experimentally to be equivalent to an oxygen uptake of 25 μl/min.

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