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The Oxidation of Manganese by Enzyme Systems

BY R. H. KENTEN AND P. J. G. MANN

Biochemistry Department, Rothamsted Experimental Station, Harpenden, Hertfordshire

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Kenten & Mann (1949, 1950) found that certain plant extracts catalysed the oxidation of bivalent manganese (Mn^{++}) by added hydrogen peroxide and showed that this catalysis was due to peroxidase systems in the plant extracts. It was suggested that *in vivo* hydrogen peroxide formed by flavoprotein enzyme systems could be used for the oxidation of Mn^{++} by peroxidase systems and colorimetric evidence of such oxidation was obtained using the xanthine-oxidase-hypoxanthine system as a source of hydrogen peroxide. In later work, Kenten & Mann (1951) demonstrated that many plant extracts, in the absence of added hydrogen peroxide, oxidized Mn^{++} and that with pea-seedling extracts the oxidation depended on the formation of hydrogen peroxide by an amine oxidase present in the extracts.

In the present work it is shown that manganese oxidation can be brought about by coupling a number of enzyme systems producing hydrogen peroxide with peroxidase systems.

MATERIALS AND METHODS

Peroxidase. This was prepared from horse-radish extracts by precipitation with $(NH_4)_2SO_4$ followed by fractional precipitation with ethanol according to the method of

Keilin & Mann (1937). The 'Purpurogallinzahl' (P.Z.) of the preparation used in the present work estimated by the method of Keilin & Mann (1937) was 250.

Catalase. This was prepared from horse liver by a method based on that of Agner (1938). The activity of the preparation was estimated by the method of Sumner & Somers (1943). The decomposition of 0.01N- H_2O_2 in 0.0067M-orthophosphate at pH 7 and 0°, by a suitable amount of catalase, was followed by iodine titration on samples taken at 0, 3, 6, 9 and 12 min. From the equation

$$k = (1/t) \log_{10} \{a/(a-x)\},$$

where a is the initial H_2O_2 concentration and $a-x$ is the concentration at time t , k values for the time intervals were calculated and the k value at zero time obtained by extrapolation. Using this value for k the catalase activity = k/g . enzyme of the preparations used varied from 5000 to 6000.

Plant amine oxidase. This was prepared from pea seedlings by the method of Kenten & Mann (1951). The dry preparation was suspended in 0.2M-pyrophosphate buffer at pH 7.5 or 0.2M-orthophosphate at pH 7 and centrifuged clear before use.

Xanthine oxidase. This was prepared from milk by the method of Dixon & Kodama (1926). The 'whey preparation' was suspended in 0.2M-pyrophosphate at pH 7.5 and centrifuged before use.

D-Amino-acid oxidase. This was prepared from sheep kidney. The method of Negelein & Brömel (1939) was followed as far as the first precipitation with $(NH_4)_2SO_4$. This precipitate was redissolved in 0.0033M-pyrophosphate

pH 8.3. The precipitate formed on addition of 15 g. $(\text{NH}_4)_2\text{SO}_4/100$ ml. was removed by centrifuging. The enzyme was precipitated by addition of a further 15 g. $(\text{NH}_4)_2\text{SO}_4/100$ ml. This was separated by centrifuging and suspended in 100 ml. 0.0033 M-pyrophosphate pH 8.3, kept at 0° overnight and centrifuged clear before use.

α -Hydroxyacid oxidase was prepared from dried leaves of brussels sprouts (*Brassica oleracea* var. *gemmifera* Zenk.). The temperature of the leaf extracts was kept below 5° throughout the preparation except during centrifuging. The final preparations were stored frozen. The small outside leaves of the axillary buds ('sprouts') were oven dried at 37°. The dried leaves were powdered in a mortar and passed through a 0.5 mm. mesh sieve. The dry powder could be stored for several weeks at room temperature with little loss of activity. For the enzyme preparation 15 g. samples were suspended in 300 ml. 0.1 M-phosphate at pH 8 and 0° and treated for a few minutes in a Townson & Mercer macerator, cooling when necessary to prevent the temperature rising above 5°. The suspension was then squeezed through mada-pollam and an α -hydroxyacid oxidase preparation was made from the extract so obtained by the procedure of Clagett, Tolbert & Burris (1949) up to the $(\text{NH}_4)_2\text{SO}_4$ fractionation. The fraction precipitating between 10 g. and 40 g. $(\text{NH}_4)_2\text{SO}_4/100$ ml. was dissolved in water and purified by treatment with $\text{Ca}_3(\text{PO}_4)_2$ gel at pH 8. The gel was prepared by the method of Singer & Kearney (1950) and had a dry weight of 20 mg./ml. The amounts of gel required varied with the different preparations. In general, 0.5–1.0 ml. gel/20 ml. enzyme solution was added and the suspension centrifuged at once. To the supernatant a further 2–5 ml. gel were added and the suspension left at 0° for 30 min. and then centrifuged. The gel was then suspended in 10 ml. 0.2 M-pyrophosphate buffer at pH 7.5 left 30 min. at 0° and then centrifuged. The clear supernatant fluid contained about 30% of the activity of the $(\text{NH}_4)_2\text{SO}_4$ fraction towards DL-sodium lactate. Clagett *et al.* (1949) found that $\text{Ca}_3(\text{PO}_4)_2$ gel adsorbed the enzyme at pH 8, but no elution was achieved with orthophosphate. The elution obtained with pyrophosphate at pH 7.5 may be due to its solvent action on the gel surface.

Manometric measurements. These were made in the Warburg apparatus at 25°. The volume of the reaction mixture was 3 ml. and KOH was present in the centre cup. The gas space was air unless otherwise stated.

EXPERIMENTAL AND RESULTS

Experiments in pyrophosphate with D-amino-acid oxidase, plant amine oxidase and xanthine oxidase

The reactions catalysed by amine oxidase (Kenten & Mann, 1951), D-amino-acid oxidase (Keilin & Hartree, 1936) and xanthine oxidase (ThurLOW, 1925) proceed according to the general equation

If the H_2O_2 formed is decomposed by catalase the apparent O_2 uptake is halved and the net reaction is

It is well known that H_2O_2 so produced may be used for secondary or coupled oxidations by peroxidase (ThurLOW, 1925; Harrison & ThurLOW,

1926). Where catalase is present and the primary reaction is proceeding according to Eqn. (2) such coupled oxidation causes increases in the O_2 uptake which may reach 100% if all the H_2O_2 produced is used in the coupled oxidations. Results obtained with D-amino-acid oxidase are shown in Fig. 1. In

Fig. 1. The oxidation of Mn^{++} by D-amino-acid oxidase systems coupled with peroxidase systems. □—□, D-amino-acid oxidase + DL-alanine + peroxidase + *p*-cresol + Mn^{++} + catalase; +—+, Mn^{++} omitted; Δ—Δ, *p*-cresol omitted; x—x, peroxidase omitted or *p*-cresol and Mn^{++} omitted. Pyrophosphate buffer pH 7.5. The dotted lines represent the theoretical uptakes.

this experiment the reaction mixtures in 0.17 M-pyrophosphate, pH 7.5, consisted of D-amino-acid oxidase, 0.2 ml. 0.1 M-DL-alanine, 100 μg. catalase and, where present, 100 μg. peroxidase, 0.3 ml. 0.001 M-*p*-cresol and 0.25 ml. 0.1 M- MnSO_4 . In the absence of Mn^{++} the O_2 uptake corresponded to slightly more than 1 atom O_2 (Eqn. 2). In presence of Mn^{++} the rate of O_2 uptake was doubled and the total uptake corresponded to 1 mol. $\text{O}_2/1$ mol. D-alanine (Eqn. 1) provided both *p*-cresol and peroxidase were also present. At the end of the experiment this reaction mixture was red, indicating accumulation of manganipyrophosphate. The fact that the O_2 uptake was doubled suggests that the H_2O_2 formed by the D-amino-acid oxidase system was used quantitatively by the peroxidase system to oxidize Mn^{++} . This was further investigated in similar experiments with amine oxidase, D-amino-acid oxidase and xanthine oxidase by not only measuring the O_2 uptakes but also the manganipyrophosphate which accumulated. The manganipyrophosphate was estimated manometrically with hydrazine (Kenten & Mann, 1949).

The results of these experiments are shown in Table 1. The amounts of putrescine, D-alanine and hypoxanthine used should give a theoretical uptake of 112 μ l. O₂ when oxidized by their respective oxidases in presence of catalase. The values obtained (A) were slightly higher than this. The addition of Mn⁺⁺ alone (B) produced small increases in the O₂ uptake particularly with the hypoxanthine-xanthine-oxidase system. The addition of *p*-cresol alone (C) had little effect on the total O₂ uptake. Where both Mn⁺⁺ and *p*-cresol were added, however, the O₂ uptakes were markedly

*Experiments in pyrophosphate with
 α -hydroxyacid oxidase*

Kenten & Mann (1951) found that many plant extracts oxidized added Mn⁺⁺ and suggested that this oxidation was a test for the presence in the extracts of enzyme systems producing H₂O₂. With some extracts addition of lactic acid increased the accumulation of oxidized manganese suggesting that during the oxidation of lactic acid by these extracts H₂O₂ was produced. It is known that certain α -hydroxyacids are oxidized by extracts of

Table 1. Oxidation of Mn⁺⁺ by oxidase systems coupled with peroxidase systems

(The basal reaction mixtures (A) consisted of oxidase, substrate, 100 μ g. catalase, 100 μ g. peroxidase in pyrophosphate at pH 7.5 or orthophosphate at pH 7. Other reaction mixtures contained, in addition, 0.25 ml. 0.1 M-MnSO₄ (B), 0.3 ml. 0.001 M-*p*-cresol (C) or both MnSO₄ and *p*-cresol (D). The oxidase substrate was added from the side arm after equilibration. When the O₂ uptake stopped the manometers were gassed with N₂ and 0.2 ml. saturated N₂H₄.H₂SO₄ was added from the side arm and the subsequent N₂ output measured.)

Enzyme	Substrate	Total O ₂ uptake (μ l.)				N ₂ output with hydrazine (μ l.)*			
		A	B	C	D	A	B	C	D
Experiments in pyrophosphate:									
Amine oxidase	Putrescine (10 μ moles)	124	136	127	278	0	8	0	107
D-Amino-acid oxidase	DL-Alanine (20 μ moles)	119	127	131	234	3	13	2	113
Xanthine oxidase	Hypoxanthine (5 μ moles)	127	156	125	237	4	7	4	63
α -Hydroxyacid oxidase	DL-Lactic acid (20 μ moles)	107	111	103	181	2	9	3	83
	Glycollic acid (5 μ moles)	102	104	103	157	4	14	3	70
Experiment in orthophosphate:									
Amine oxidase	Putrescine (5 μ moles)	59	59	66	115	1	6	1	81

* Corrected for 105% recovery (see Kenten & Mann, 1949.)

increased. The values obtained with the D-amino-acid oxidase and xanthine oxidase systems agreed with the theoretical 224 μ l. O₂ required by Eqn. (1), but were significantly above this with the amine oxidase system. The doubling of the O₂ uptake is due to the fact that the H₂O₂ produced in the basal reaction is not decomposed by the catalase present but is used by the peroxidase system to oxidize the added Mn⁺⁺.

If all the H₂O₂ is used to oxidize Mn⁺⁺ and the oxidized manganese accumulated quantitatively then an output of 112 μ l. N₂ would be expected on subsequent addition of hydrazine. The N₂ outputs with hydrazine using the amine oxidase and amino-acid oxidase systems agree closely with this theoretical value. The low value with the xanthine oxidase system is due, at least in part, to the fact that manganipyrophosphate oxidizes the uric acid which is formed by the action of the xanthine oxidase on the hypoxanthine, and thereby is itself reduced. It is clear from these results that H₂O₂ formed by enzyme systems can be used quantitatively by peroxidase for the oxidation of Mn⁺⁺.

the leaves of many plants. Kolesnikov (1948a) found that extracts of barley leaves oxidized glycollic acid and Clagett *et al.* (1949) showed the presence in plant leaves of an α -hydroxyacid oxidase catalysing the oxidation of both lactic and glycollic acids. More recent work, Tolbert & Burris (1950), indicates that different enzymes may be responsible for the oxidation of lactic and glycollic acids. While considerations of the total O₂ uptake suggest that H₂O₂ is formed during the oxidations. Tolbert & Burris (1950) were unable to demonstrate its presence and, like Kolesnikov (1948b) suggested that an organic peroxide might be formed. Since the preliminary experiments of Kenten & Mann (1951) suggested that the α -hydroxyacid oxidase system might be suitable for the demonstration of coupled oxidation of Mn⁺⁺, preparations of this enzyme were tested under the conditions of Table 1. The amounts of substrate were chosen so that in absence of coupled oxidation the theoretical uptake was 112 μ l. O₂ with purified enzyme preparations; the uptake with L-lactic acid is 1 atom O/mol. L-lactic acid, and with glycollic acid 1 mol. O₂/mol. glycollic acid (Clagett *et al.* 1949). The uptakes with

both substrates (A) were slightly less than the theoretical value, particularly with glycollic acid. With glycollic acid the oxidation proceeds in two stages (Clagett *et al.* 1949). The oxidation of glycollic acid to glyoxylic acid which constitutes the first stage is rapid in comparison with the further oxidation of glyoxylic acid to formic acid. Addition of Mn^{++} and *p*-cresol (D) produced marked increases in the O_2 uptakes which with lactic acid approached the theoretical doubling while with glycollic acid the increase was approximately 50 %. The N_2 outputs with hydrazine show that these increases in O_2 uptake were due to the oxidation of Mn^{++} and accumulation of manganipyrophosphate. It is clear therefore that a peroxide which can be used by peroxidase to oxidize Mn^{++} , and which is decomposed by catalase, is formed during the oxidation of both lactic and glycollic acids by α -hydroxyacid oxidase.

*Experiments in orthophosphate
with amine oxidase*

Accumulation of manganese oxidation product. In previous work (Kenten & Mann, 1949, 1950) where the oxidation of Mn^{++} was brought about by peroxidase systems with added H_2O_2 , it was found that in orthophosphate media the manganese oxidation product reacted with the H_2O_2 as rapidly as it was formed and therefore did not accumulate. In media where stable manganic complexes could be formed, such as pyrophosphate, the oxidation product accumulated though not quantitatively. To explain these results it was shown that manganipyrophosphate reacts less rapidly than MnO_2 with H_2O_2 . It was found that the amount of accumulated manganipyrophosphate, calculated as a percentage of that theoretically possible, increased with decreasing H_2O_2 concentration, and it was suggested that at sufficiently low H_2O_2 concentrations all the H_2O_2 would be used in the accumulation of manganipyrophosphate. It has been shown in the experiments of Table 1 that where enzyme systems are used to provide this low concentration of H_2O_2 , manganipyrophosphate may accumulate quantitatively. With these systems accumulation of manganese oxidation products can also be demonstrated in orthophosphate media (Table 1). The doubling of the O_2 uptake produced by the addition of Mn^{++} suggested that none of the manganese oxidation product reacts with the H_2O_2 under these conditions. It was found, however, that the hydrazine method was unsuitable to prove that the accumulation was quantitative. The oxidation product, which was present as a red-brown precipitate, reacted much more slowly than manganipyrophosphate or hydrated MnO_2 with the hydrazine, but finally gave a total output greater than that required by theory. The reason for this is not

yet clear since MnO_2 reacts quantitatively with hydrazine in orthophosphate media.

Experiments with catalytic amounts of manganese. In the preceding experiments designed to demonstrate quantitative accumulation of manganese oxidation product, Mn^{++} was added in amounts more than equivalent to the H_2O_2 formed by the primary enzyme system, and was therefore present in relatively high concentration. Attempts were made to show that Mn^{++} was oxidized, when present in low concentrations, by adding to the reaction

Fig. 2. The oxidation of Mn^{++} by an amine oxidase system coupled with a peroxidase system demonstrated by the oxidation of added oxalic acid. $\square$ — $\square$, Amine oxidase + cadaverine + peroxidase + catalase + *p*-cresol + Mn^{++} + oxalic acid; $\triangle$ — $\triangle$, *p*-cresol omitted; $\times$ — $\times$, Mn^{++} omitted; $\circ$ — $\circ$, *p*-cresol and Mn^{++} omitted. Orthophosphate buffer pH 5. The dotted lines represent the theoretical O_2 uptakes.

mixtures compounds likely to reduce the oxidized manganese as fast as it was formed. The oxidation of such small amounts of Mn^{++} with accumulation of the oxidation product would produce little increase in the O_2 uptake. If, however, the Mn^{++} entered a cycle of oxidation and reduction, then such amounts would be capable of producing large increases in the O_2 uptake. This was tested with 110 $\mu g.$ Mn^{++} using the amine oxidase system as source of H_2O_2 and oxalic acid as reductant of the oxidized manganese. The results of one such experiment are shown in Fig. 2. Reaction mixtures in 0.067 M-orthophos-

phate at pH 5 consisted of amine oxidase (4 mg.), 0.2 ml. 0.05M-cadaverine, 100 μ g. catalase, 100 μ g. peroxidase, 0.3 ml. 0.001M-*p*-cresol, 0.2 ml. 0.01M- MnSO_4 and 0.5 ml. 0.1M-sodium oxalate. The cadaverine was added from the side arm after equilibration. As expected, in the presence of both Mn^{++} and *p*-cresol, an increase in O_2 uptake was observed which was much greater than that required for the complete oxidation of the added Mn^{++} to MnO_2 (22.4 μ l. O_2). It is clear therefore that the Mn^{++} undergoes a cycle of oxidation and reduction, and that the observed increase in O_2 uptake is mainly due to the oxidation of the oxalic acid brought about by the oxidized manganese; but the results obtained suggest the possibility that reactions other than the coupled oxidation of the Mn^{++} by the enzyme system and reduction of the oxidation product by the oxalic acid take place. Thus if all the H_2O_2 produced in the oxidation of the cadaverine was used for oxalic acid oxidation via the Mn^{++} , a total O_2 uptake of 224 μ l. (1 mol. O_2 /mol. cadaverine) would be expected (Eqn. 1). At the end of the experiment the O_2 uptake in the reaction mixture containing Mn^{++} and *p*-cresol was not complete but already exceeded 224 μ l. Also, in the absence of *p*-cresol, Mn^{++} alone caused a marked increase in the O_2 uptake in the later stages of the experiment. These observations have led to a separate investigation of reactions involving peroxidase systems, Mn^{++} and oxalic acid.

DISCUSSION

The results of the present work show that, in presence of a system which maintains a suitably low concentration of H_2O_2 , peroxidase systems can bring about oxidation of Mn^{++} with quantitative accumulation of the oxidation product. In contrast to previous results with added H_2O_2 (Kenten & Mann, 1950) accumulation of the Mn oxidation product can be demonstrated in orthophosphate as well as in pyrophosphate media. It is clear from these results that, provided the H_2O_2 concentration is sufficiently low, the rate of reduction of the oxidized Mn by the H_2O_2 is negligible. Under these conditions all the oxidized Mn would therefore be available in the plant for the oxidation of metabolites other than H_2O_2 , as postulated by Kenten & Mann (1949), and the Mn^{++} would undergo a cycle of oxidation and reduction as demonstrated in the present work with oxalic acid as a reducing agent.

This oxidation of Mn^{++} has not yet been demonstrated in the plant *in vivo* under normal conditions of manganese supply. In preliminary experiments with plants grown in water culture with high manganese concentrations, evidence has been obtained of the accumulation of oxidized Mn in the

tissues. Such accumulation can generally be shown in the pea plant (*Pisum sativum* L.) with concentrations of Mn^{++} of the order of 50 p.p.m. in the culture solution. Despite the sensitivity of the test used it has not yet been possible to demonstrate such accumulation regularly with more normal conditions of Mn supply. The results suggest, however, that Mn oxidation does occur within the plant *in vivo* and, in view of previous results with plant extracts (Kenten & Mann, 1951), it is reasonable to postulate that this oxidation is brought about by the peroxidase system. It has already been shown that the oxidation takes place *in vitro* with physiological concentrations of Mn^{++} . At these concentrations of Mn^{++} *in vivo* it is possible that the Mn oxidation product is reduced by plant metabolites as fast as it is formed, and that only under conditions of Mn toxicity is the rate of oxidation of Mn greater than the rate of reduction of the oxidation product by plant metabolites. This suggests the possibility that the symptoms of Mn toxicity may be due, at least in part, to the deposition of higher oxides of manganese in the tissues. Studies with enzyme preparations suggest that Mn^{++} ions may function as activators of many enzyme systems *in vivo*. In general, such effects are not specific for Mn^{++} ions, but to a greater or lesser extent are shared by other bivalent cations. It has not yet been ascertained how far the activity of such enzymes is affected in Mn deficiency, but it may prove that *in vivo* Mn^{++} ions function as specific activators of one or more enzyme systems. It is therefore not suggested that the effects of Mn on plant metabolism depend entirely on the oxidation-reduction cycle postulated. However, an effect of plant metabolism due to such a cycle would be one of some specificity for Mn since, of the bivalent cations tested, peroxidase systems only catalyse the oxidation of Fe^{++} and Mn^{++} .

Lastly it should be pointed out that, as suggested by Kenten & Mann (1951), the oxidation of Mn^{++} by peroxidase systems serves as a useful test for enzyme systems producing H_2O_2 . In the present work it is shown by this means that α -hydroxyacid oxidase produces H_2O_2 when catalysing the oxidation of lactic and glycollic acids. This finding has now been confirmed by more conventional methods (Kenten & Mann, 1952).

SUMMARY

1. The oxidation of bivalent manganese (Mn^{++}) can be brought about by enzyme systems producing hydrogen peroxide such as D-amino-acid oxidase, xanthine oxidase and plant amine oxidase systems coupled with peroxidase systems.

2. With high Mn^{++} concentrations the oxidation was demonstrated by the increased oxygen uptake

and by estimation of the manganese oxidation product with hydrazine. The oxidation product accumulates in both pyrophosphate and orthophosphate media.

3. With low Mn^{++} concentrations the oxidation was demonstrated by the increased oxygen uptake of the system in presence of oxalic acid. Under these

conditions the manganese is involved in a cycle of oxidation and reduction.

4. The oxidation of Mn^{++} by peroxidase systems can be used as a test for hydrogen peroxide. By this test it has been shown that plant α -hydroxyacid oxidase produces hydrogen peroxide while catalysing the oxidation of L-lactic and glycollic acids.

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Hydrogen Peroxide Formation in Oxidations Catalysed by Plant α -Hydroxyacid Oxidase

BY R. H. KENTEN AND P. J. G. MANN

Biochemistry Department, Rothamsted Experimental Station, Harpenden, Hertfordshire

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Kolesnikov (1948a) found that extracts of barley leaves oxidized glycollic acid, and Clagett, Tolbert & Burris (1949) showed the presence in leaves of many plants of an α -hydroxyacid oxidase catalysing the oxidation of lactic, glycollic and glyoxylic acids. More recent work (Tolbert & Burris, 1950) indicates that different enzymes may be responsible for the oxidation of these acids. While considerations of the total oxygen uptake suggested that hydrogen peroxide is formed during the oxidations, Tolbert & Burris (1950) were unable to demonstrate its presence, and like Kolesnikov (1948b) suggested that an organic peroxide might be formed. Kenten & Mann (1952) demonstrated the oxidation of manganese by flavoprotein enzyme systems coupled with peroxidase systems. It was found that α -hydroxyacid oxidase could replace the known flavoprotein enzymes in producing systems capable of oxidizing manganese, and it was concluded that this was due to the formation of hydrogen peroxide by α -hydroxyacid oxidase systems. In the present work this hydrogen peroxide formation is demonstrated by more conventional methods and its influence on the course of the oxidations is investigated.

MATERIALS AND METHODS

α -Hydroxyacid oxidase. This was prepared from leaves of tobacco (*Nicotiana tabacum* L.). The leaves were minced in a cooled domestic meat mincer and squeezed through mado-pollam. The temperature of the sap was kept below 5° during the subsequent procedure except during centrifuging. Sufficient 2M- K_2HPO_4 was added to the sap to give a final concentration of 0.1M. The procedure of Clagett *et al.* (1949) was then followed up to and including the first $(NH_4)_2SO_4$ fractionation. The active precipitate from this fractionation was dissolved in water (25 ml./100 ml. original sap) and either dialysed against 0.01M-phosphate buffer at pH 7 overnight in the refrigerator or treated with $Ca_3(PO_4)_2$ gel to reduce the catalase content. This treatment was based on the observation of Agner (1938) that catalase is adsorbed by the gel at pH 5.5. The gel was made by the method of Singer & Kearney (1950) and had a dry weight of 20 mg./ml. The enzyme solution was treated successively with small amounts of gel at pH 5.5. After each addition the suspension was left 30 min. at 0° and then centrifuged. The supernatant was then tested for α -hydroxyacid oxidase and catalase. The amounts of gel required varied with the different enzyme preparations. In one case 20 ml. enzyme solution were treated with 4 ml. of gel followed by two treatments with 1 ml. The catalase activity was then 2%, and the α -hydroxyacid oxidase activity 50%, of that before gel treatment. In