The Action of Peroxidase Systems on Ferrocyanide, Molybdate, Tungstate and Vanadate

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Kenten & Mann (1950) showed that plant peroxidase preparations in the presence of certain phenolic substrates catalyse the oxidation of Mn^{++} by hydrogen peroxide. Evidence was obtained that the Mn^{++} reduces an intermediate oxidation product of the phenolic substrate and thereby is itself oxidized. The oxidizing capabilities of the system peroxidase + phenolic substrate + hydrogen peroxide towards inorganic compounds have now been further investigated. It is shown that the system oxidizes ferrocyanide, and evidence suggesting the oxidation of molybdate, tungstate and vanadate is reported.

MATERIALS AND METHODS

Peroxidase preparations were obtained from horse-radish by the method of Keilin & Mann (1937). The 'Purpuro-gallinzahl' (P.Z.) of these preparations was determined by the method of Keilin & Mann (1937).

Hydrogen peroxide. In the experiments with molybdate, tungstate and vanadate, approximately $0.05M-H_2O_2$ was used, and for the manometric experiments with ferrocyanide approximately $0.025M-H_2O_2$ in $0.0105M-NaHCO_3$. Fresh solutions were prepared for each experiment. The $0.025M-H_2O_2$ in $NaHCO_3$ was estimated by $KMnO_4$ titration and the $0.05M-H_2O_2$ manometrically with MnO_2 in acid solution (Kenten & Mann, 1950).

Ammonium permolybdate was prepared by treatment of 100 vol. H_2O_2 with molybdate according to the method of Rosenheim, Hakki & Krause (1932). The peroxidic oxygen was estimated by thiosulphate titration of the I_2 liberated from KI in acid solution by the permolybdate. Values obtained in various preparations varied from 7.8 to 9.6%. Rosenheim *et al.* (1932) found 7.1–7.5% peroxidic oxygen.

Uranium peroxide was prepared by treatment of uranyl nitrate with H_2O_2 at $40-50^\circ$ according to the method of Watt, Achorn & Marley (1950). Peroxidic oxygen varied from 9.2 to 9.4%. Watt *et al.* (1950) found 9.0%.

Manometric measurements were carried out in the Warburg apparatus at 25° . The experiments with molybdate, tungstate and vanadate were made in phosphate buffers; the volume of the reaction mixture was 3 ml., KOH was present in the centre cup and the gas space was air. The ferrocyanide experiments were made in bicarbonate media; the volume of the reaction mixture was 4.5 ml., KOH was omitted from the centre cup and the gas space was 95% N_2 + 5% CO_2 .

EXPERIMENTAL AND RESULTS

Oxidation of ferro- to ferricyanide by peroxidase systems

Colorimetric evidence. Reaction mixtures consisting of horse-radish peroxidase of P.Z. 105 (40 $\mu g.$ in 0.5 ml. water), 1 ml. $0.025M-K_4Fe(CN)_6$, 1 ml.

m -pyrophosphate at pH 7 and 0.3 ml. $0.1M$ -phenolic substrate were made up in test tubes and 0.3 ml. $0.05M-H_2O_2$ was added. Control experiments were made in which each of the reactants was omitted in turn, or in which both phenolic substrate and peroxidase were omitted. With the complete system, using either phenol, p -cresol or o -cresol, the reaction mixture within 1 min. developed a strong yellow colour indicating ferricyanide formation. With peroxidase heated for 15 min. in a boiling-water bath or with H_2O_2 in absence of peroxidase little or no oxidation of ferrocyanide could be observed within this time, but with peroxidase in absence of phenolic substrate a faint yellow colour had developed in 1 min. The results suggested that peroxidase alone catalyses the oxidation of ferro- to ferri-cyanide by H_2O_2 , but that the system formed by peroxidase, together with a suitable phenolic substrate, was a much more active catalyst of the reaction.

Manometric evidence. The oxidation of ferro- to ferri-cyanide is accompanied by the consumption of H^+ , and may therefore be followed manometrically by measurement of CO_2 uptake in bicarbonate media in an atmosphere of $N_2 + CO_2$ (cf. Haas, 1937; Quastel & Wheatley, 1938).

Equations 1 and 2 show that for each molecule of H_2O_2 used in the oxidation of ferrocyanide two molecules of CO_2 will be taken up.

Reaction mixtures consisted of 3 $\mu g.$ peroxidase of P.Z. 480, 0.6 ml. of $0.05M-K_4Fe(CN)_6$, 0.45 ml. of $0.1M$ - p -cresol in $0.0105M-NaHCO_3$; 0.2 ml. of $0.025M-H_2O_2$ in $0.0105M-NaHCO_3$ was added from the side arm. The total volume of the reaction mixtures was 4.5 ml. The gas space was filled with 95% N_2 + 5% CO_2 . Under these conditions the reaction of the medium after equilibration was about pH 7. Control experiments were made in which either p -cresol or peroxidase was omitted.

A typical set of results is shown in Fig. 1. On the addition of the H_2O_2 from the side arm a rapid uptake of CO_2 took place, where both peroxidase and p -cresol were present. The observed uptake, which was complete in about 10 min., varied in different experiments between 90 and 110% of theory based on the H_2O_2 added. In the control experiments in absence

of peroxidase the initial rate of uptake was about 15 % and in absence of *p*-cresol about 30 % of the rate where the complete system was present. The results show that peroxidase in presence of *p*-cresol catalyses the oxidation of ferrocyanide by H_2O_2 . In further experiments it was found that phenol could be

been heated for 15 min. in a boiling-water bath, had no effect on the rate of CO_2 uptake. Comparison of Figs. 1 and 2 shows that 3 μg . of the peroxidase preparation in presence of *p*-cresol catalyses the oxidation of ferrocyanide by H_2O_2 at about the same rate as 16 μg . of the peroxidase preparation in absence of *p*-cresol.

Fig. 1. The effect of peroxidase and *p*-cresol on the oxidation of ferrocyanide by H_2O_2 . Δ — Δ , peroxidase + *p*-cresol + ferrocyanide; O—O, peroxidase + ferrocyanide; +—+, ferrocyanide; x—x, peroxidase + *p*-cresol. H_2O_2 added to all reaction mixtures. The dotted line shows the theoretical uptake.

substituted for *p*-cresol, but negative results were obtained with pyrogallol and catechol. Similarly, Kenten & Mann (1950) found that the oxidation of Mn^{++} by peroxidase systems could be demonstrated with phenol or *p*-cresol, but not with pyrogallol or catechol as phenolic substrates.

The results of Fig. 1 also suggest that peroxidase in absence of *p*-cresol may catalyse the oxidation of ferrocyanide by H_2O_2 though at a much slower rate than when *p*-cresol also is present. This was shown more clearly by using larger amounts of peroxidase (Fig. 2). The reaction mixtures were made up as before except that *p*-cresol was omitted and the peroxidase additions varied between 0 and 32 μg . of P.Z. 480. A linear relationship was found between the increased rate of CO_2 uptake and peroxidase added up to 16 μg . of P.Z. 480. The rate could be still further increased with larger amounts of peroxidase, though here the relationship was not linear. Control experiments showed that peroxidase, which had

Fig. 2. The effect of peroxidase on the oxidation of ferrocyanide by H_2O_2 . Peroxidase added, O—O, 32 μg .; $\square$ — $\square$, 16 μg .; $\bullet$ — $\bullet$, 8 μg .; x—x, 4 μg .; Δ — Δ , 0 μg .; +—+, 32 μg ., heated for 15 min. at 100°. The dotted line shows the theoretical uptake.

Oxidation of molybdate, vanadate and tungstate

Effects of molybdates, tungstates, and vanadates on the oxidation of Mn^{++} by peroxidase systems. It has previously been shown (Kenten & Mann, 1949, 1950) that the oxidation of manganese by peroxidase systems can be followed manometrically by measuring the evolution of O_2 produced when the manganese oxidation product reacts stoichiometrically with the H_2O_2 . Assuming the manganese oxidation product is Mn_2O_3 , the reactions can be represented by the equations:

Under suitable conditions complete decomposition of added H_2O_2 is observed. If insufficient Mn^{++} is

present to reduce the oxidized phenolic substrate, this is further oxidized to products which Mn^{++} cannot reduce. When Mn^{++} is the limiting factor the evolution of O_2 rapidly ceases owing to the complete loss by irreversible oxidation of the phenolic substrate. In these circumstances the addition of small amounts of molybdates, tungstates or vanadates markedly increased both the rate of evolution and the total O_2 output. Results illustrating this effect with ammonium molybdate are shown in Fig. 3.

Fig. 3. The effect of ammonium molybdate on the oxidation of Mn^{++} by the system peroxidase, *p*-cresol and H_2O_2 . Peroxidase ($2 \mu g.$) + *p*-cresol + Mn^{++} ($5.5 \mu g.$) with the following concentrations of ammonium molybdate: $\blacksquare$ — $\blacksquare$, $9.6 \times 10^{-6} M$; $\otimes$ — $\otimes$, $4.8 \times 10^{-6} M$; $\square$ — $\square$, $2.4 \times 10^{-6} M$; $\triangle$ — $\triangle$, $1.2 \times 10^{-6} M$; $\bullet$ — $\bullet$, 0. Controls with $9.6 \times 10^{-6} M$ -ammonium molybdate; $\circ$ — $\circ$, *p*-cresol omitted; $\times$ — $\times$, peroxidase omitted; $+$ — $+$, Mn^{++} omitted. The dotted line shows the theoretical output.

Reaction mixtures consisted of $2 \mu g.$ peroxidase of P.Z. 250, 0.2 ml. of 0.005 *M*-*p*-cresol, 0.1 ml. 0.001 *M*- $MnSO_4$ ($5.5 \mu g.$ Mn^{++}), and varying amounts of ammonium molybdate (2.4 – $192 \mu g.$ Mo) in 0.033 *M*-orthophosphate at pH 7; 0.2 ml. of 0.05 *M*- H_2O_2 was added from the side arm after equilibration. Control reaction mixtures were also made in which either $MnSO_4$, ammonium molybdate or *p*-cresol were omitted. In absence of molybdate the total O_2 output was about $20 \mu l.$; where molybdate was also present, both the rate and total O_2 output were markedly increased even at the lowest molybdate

concentration of $1.2 \times 10^{-6} M$ ($2.4 \mu g.$ Mo). With the highest molybdate concentration ($9.6 \times 10^{-6} M$) the O_2 output was complete in about 30 min. and was equivalent to decomposition of about 90% of the H_2O_2 added. Where $MnSO_4$ was omitted from the reaction mixtures the decomposition of H_2O_2 produced by molybdate at concentrations of 10^{-5} to $10^{-6} M$ was negligible. The decomposition of H_2O_2 observed in the presence of both $MnSO_4$ and molybdate was therefore not merely a summation of the separate effects of molybdate and $MnSO_4$. Similar effects were obtained with tungstates and vanadates in place of molybdates but not with borate, chromate and uranate.

Fig. 4. The effect of ammonium molybdate on the oxidation of Mn^{++} by H_2O_2 . Mn^{++} ($5.5 \mu g.$) with the following concentrations of ammonium molybdate: $\blacksquare$ — $\blacksquare$, $7.5 \times 10^{-5} M$; $\square$ — $\square$, $3.7 \times 10^{-5} M$; $\triangle$ — $\triangle$, $9.6 \times 10^{-6} M$; $\circ$ — $\circ$, $1.2 \times 10^{-6} M$; $\bullet$ — $\bullet$, 0. $\times$ — $\times$, control with $7.5 \times 10^{-5} M$ -ammonium molybdate, Mn^{++} omitted. The dotted line represents the theoretical output.

The oxidation of Mn^{++} by H_2O_2 in presence of molybdates, tungstates and vanadates

In the absence of *p*-cresol or peroxidase, $MnSO_4$ and molybdate together, at the concentrations used in the previous experiment, caused a decomposition of H_2O_2 which was slow in comparison with that produced when the enzyme and its substrate were also present, but much greater than the sum of the effects of $MnSO_4$ and molybdate separately. The rate of this decomposition of H_2O_2 by $MnSO_4$ and molybdate in absence of peroxidase and *p*-cresol could be increased by increasing the concentration of molybdate (Fig. 4).

Reaction mixtures consisted of 0.1 ml. 0.001M- MnSO_4 (5.5 μg . Mn^{++}) and varying amounts of ammonium molybdate (2.4–150 μg . Mo) in 0.033M-orthophosphate at pH 7; 0.2 ml. 0.05M- H_2O_2 was added from the side arm after equilibration. The results suggest that if the concentration of molybdate is increased to a sufficiently high level, the rate of decomposition of H_2O_2 may be as rapid as that obtained with small amounts of molybdate in the presence of peroxidase and *p*-cresol. But at the highest concentration of molybdate used ($7.5 \times 10^{-5}\text{M}$) the rate was about half that obtained at one-eighth the concentration when peroxidase and *p*-cresol were also present (Fig. 1). Similar results were obtained using tungstates or vanadates in place of molybdates, but not with borate, chromate or uranate.

It is known that H_2O_2 oxidizes molybdates, tungstates, vanadates, chromates and uranates to peracids according to the general equation

The oxidation takes place readily at room temperature. The experiments in the next section show that permolybdates oxidize Mn^{++} and suggest that the interaction between molybdates and Mn^{++} in the decomposition of H_2O_2 is due to the formation of permolybdates. Since peroxidase and *p*-cresol catalyse the molybdate effect at low concentrations of molybdate it is suggested that peroxidase and *p*-cresol catalyse the formation of permolybdates under these conditions.

Oxidation of Mn^{++} by permolybdates

Manometric evidence. MnO_2 and manganipyrophosphate rapidly decompose aqueous solutions of permolybdate with the evolution of O_2 . If permolybdate oxidizes Mn^{++} , O_2 should be evolved as a result of the reaction and under conditions where the oxidized manganese does not accumulate the O_2 output is a measure of the Mn^{++} oxidized. Assuming the oxidation is to the manganic state the reaction can be represented as follows:

This was tested in reaction mixtures consisting of 0.1 ml. 0.001M- MnSO_4 in 0.033M-orthophosphate at pH 7; varying amounts of a solution of ammonium permolybdate were added from the side arm after equilibration.

In the presence of MnSO_4 a rapid evolution of O_2 took place, the total O_2 output in each case being approximately equivalent to the peroxidic oxygen in the permolybdate (Fig. 5). This is consistent with the hypothesis that permolybdate oxidizes Mn^{++} .

Isolation of MnO_2 . Kenten & Mann (1949), while studying the oxidation of Mn^{++} by peroxidase

systems, found that in pyrophosphate solutions at high concentrations of Mn^{++} the rate of oxidation of Mn^{++} was greater than its rate of reduction by the H_2O_2 , and manganipyrophosphate therefore accumulated. By the addition of alkali the manganipyrophosphate was converted to MnO_2 which was isolated. Under similar conditions it has been possible to isolate small amounts of MnO_2 from large-scale reaction mixtures of MnSO_4 and ammonium permolybdate, thus obtaining direct proof that permolybdate oxidizes Mn^{++} .

Fig. 5. The effect of Mn^{++} on the decomposition of ammonium permolybdate. Mn^{++} (5.5 μg .) with the following amounts of ammonium permolybdate (peroxidic oxygen 9.4%): ■—■, 4.8 mg.; □—□, 3.2 mg.; △—△, 1.6 mg.; ○—○, control with 4.8 mg. ammonium permolybdate, Mn^{++} omitted. The dotted lines show the theoretical output.

To a reaction mixture containing 40 ml. of 0.5M- MnSO_4 and 300 ml. of M-pyrophosphate at pH 7, 1.6 g. of ammonium permolybdate (8.2% peroxidic oxygen) in 8 ml. of water were added. The colour of the reaction mixture which was initially pale yellow turned through orange to deep red within 5 min. The reaction mixture was allowed to stand for 4 hr. and a white precipitate which had formed was then centrifuged off. The supernatant was adjusted to pH 10 (glass electrode) and the MnO_2 which precipitated was isolated and analysed by the procedure of Kenten & Mann (1949). The yield of MnO_2 (62 mg.; valency 4.03) was 17% based on the permolybdate added.

DISCUSSION

The results of the present work show that peroxidase in presence of suitable phenolic substrates catalyses the oxidation of ferrocyanide by hydrogen peroxide and possibly that of molybdates, vanadates and

tungstates, though in the latter cases direct proof of the oxidation was not obtained. Molybdate, vanadate and tungstate catalyse the oxidation of Mn^{++} by peroxidase systems, and it is inferred that this catalysis is brought about through the oxidation of these complex ions to peracids.

According to Jahr (1941) the oxidation of sodium molybdate by hydrogen peroxide proceeds according to the following equations:

In the present work, in agreement with eqn. 9, a carbon dioxide uptake was found when hydrogen peroxide was added to sodium molybdate in bicarbonate buffer at pH 7 in presence of nitrogen-carbon dioxide. Peroxidase and *p*-cresol had little effect on the rate of this uptake. In such experiments a relatively high concentration of sodium molybdate, e.g. 10^{-2} to $10^{-3}M$, has to be employed to obtain a reasonable carbon dioxide uptake, whereas the indirect evidence that peroxidase catalyses permolybdate formation was obtained at concentrations of 10^{-5} to $10^{-6}M$ -ammonium molybdate.

In absence of phenolic substrate peroxidase catalyses the oxidation of ferrocyanide by the hydrogen peroxide, though at a much reduced rate, but no evidence has been found that peroxidase alone catalyses the oxidation of molybdates, vanadates and tungstates by hydrogen peroxide.

These results, in conjunction with those previously obtained on manganese oxidation (Kenten & Mann, 1950), are of possible significance in considering the functions both of peroxidase and of certain micro-nutrients in plant metabolism. It has been suggested that tyrosinase may act as a terminal oxidase in plant respiration, the quinone formed by the action of the oxidase acting as a hydrogen acceptor (e.g. Boswell & Whiting, 1938; Baker & Nelson, 1943). Since quinones oxidize coenzyme I (Dixon & Zervas, 1940) and coenzyme II (Kubowitz, 1937), tyrosinase could thus act as a link between oxygen and dehydrogenases requiring these coenzymes. In a similar manner peroxidase could link enzymes producing hydrogen peroxide to such dehydrogenase systems. It is possible that manganese, molybdates, vanadates and tungstates can form a link between peroxidase and plant metabolites and dehydrogenase systems other than those dependent on coenzymes I and II, and thus extend the range of peroxidase activity. Chance (1949) has shown that peroxidase oxidizes ferrocytochrome *c*, which suggests a possible connexion between peroxidase and

plant respiration dependent on carriers containing ferrous iron as the active group. The results of the present work suggest that such carriers need not necessarily be haemoproteins. In this connexion it is of interest that laccase catalyses the oxidation of ferrocyanide by oxygen (Gregg & Miller, 1940).

The available evidence indicates that the main biological importance of molybdenum in plant metabolism is in connexion with the nitrogen cycle, e.g. in the fixation of atmospheric nitrogen by the free-living organisms *Azotobacter* and *Clostridia*, and the root nodule bacteria *Rhizobia* (Bortels, 1930; Horner, Burk, Allison & Sherman, 1942), and in the process of nitrate assimilation (Hewitt & Jones, 1947; Hewitt, Jones & Williams, 1949; Mulder, 1948). It is possible, however, that the biological activity of molybdenum is not restricted to its effect on nitrogen metabolism and that its effect on nitrate assimilation and nitrogen fixation is not directly on the reduction processes involved, but on the associated oxidative reactions which supply the energy required by these processes. Hewitt, Agarwala & Jones (1950) find that molybdenum deficiency consistently causes a striking reduction in the apparent ascorbic acid content of plants, and discuss the possibility that ascorbic acid may function in plants as an agent in nitrate reduction.

The oxidation of iron, manganese and molybdate in plants *in vivo* by peroxidase systems depends on the formation of hydrogen peroxide by the plant tissue. Work in progress shows that manganese is oxidized by plant extracts and that such oxidation depends on the formation of hydrogen peroxide by the extracts. In fact, this oxidation of manganese forms a useful test for the presence of enzyme systems producing hydrogen peroxide.

SUMMARY

1. Plant peroxidase catalyses the oxidation of ferrocyanide by hydrogen peroxide. The system formed by peroxidase, together with a suitable phenolic substrate, is a much more effective catalyst of the reaction.

2. Under certain conditions the rate of oxidation of manganese by peroxidase systems is markedly increased by adding molybdates, tungstates or vanadates.

3. Evidence has been obtained that this effect is due to catalysis by peroxidase systems of the oxidation of molybdates, tungstates and vanadates to the corresponding peroxy compounds by hydrogen peroxide.

REFERENCES

- Baker, D. & Nelson, J. M. (1943). *J. gen. Physiol.* **26**, 269.
 Bortels, H. (1930). *Arch. Mikrobiol.* **1**, 333.
 Boswell, J. G. & Whiting, G. C. (1938). *Ann. Bot., N.S.*, **11**, 847.
 Chance, B. (1949). *Science*, **109**, 204.
 Dixon, M. & Zervas, L. G. (1940). *Biochem. J.* **34**, 371.
 Gregg, D. C. & Miller, W. H. (1940). *J. Amer. chem. Soc.* **62**, 1374.
 Haas, E. (1937). *Biochem. Z.* **291**, 79.
 Hewitt, E. J., Agarwala, S. C. & Jones, E. W. (1950). *Nature, Lond.*, **166**, 1119.
 Hewitt, E. J. & Jones, E. W. (1947). *J. Pomol.* **23**, 254.
 Hewitt, E. J., Jones, E. W. & Williams, A. H. (1949). *Nature, Lond.*, **163**, 681.
 Horner, C. K., Burk, D., Allison, F. E. & Sherman, M. S. (1942). *J. agric. Res.* **65**, 173.
 Jahr, K. F. (1941). *Chem. Zbl.* **1**, 184.
 Keilin, D. & Mann, T. (1937). *Proc. roy. Soc. B*, **122**, 119.
 Kenten, R. H. & Mann, P. J. G. (1949). *Biochem. J.* **45**, 255.
 Kenten, R. H. & Mann, P. J. G. (1950). *Biochem. J.* **46**, 67.
 Kubowitz, F. (1937). *Biochem. Z.* **292**, 221.
 Mulder, E. G. (1948). *Plant & Soil*, **1**, 94.
 Quastel, J. H. & Wheatley, A. H. M. (1938). *Biochem. J.* **32**, 936.
 Rosenheim, A., Hakki, M. & Krause, O. (1932). *Z. anorg. Chem.* **209**, 175.
 Watt, G. W., Achorn, S. L. & Marley, J. L. (1950). *J. Amer. chem. Soc.* **72**, 3341.

The Assimilation of Amino-Acids by Bacteria

13. THE EFFECT OF CERTAIN AMINO-ACIDS ON THE ACCUMULATION OF FREE GLUTAMIC ACID BY *STAPHYLOCOCCUS AUREUS*: EXTRACELLULAR PEPTIDE FORMATION

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If washed suspensions of *Staphylococcus aureus* (*Micrococcus pyogenes* var. *aureus*) are incubated in buffered salt solution containing glutamic acid and glucose, the amino-acid enters and accumulates in the free state within the cells (Gale, 1947). The passage of glutamic acid into the cells is energy-linked and can be uncoupled from glycolysis by inhibitors such as sodium azide, 2:4-dinitrophenol, aureomycin and 8-hydroxyquinoline (Gale, 1949, 1951*a*; Gale & Paine, 1951). If a complete mixture of natural amino-acids is added to the external medium, the accumulation of free glutamic acid within the cells ceases, and glutamic acid becomes incorporated within the cellular material in a combined form from which it can be liberated by acid hydrolysis (Gale, 1951*b*). The addition of incomplete mixtures of amino-acids to the external medium results in a reduction in the rate of accumulation of free glutamic acid within the cells (Gale, 1951*b*), and the present communication deals with the mechanism of this inhibition.

EXPERIMENTAL

Organism. The organism used throughout this work was *Staphylococcus aureus* Duncan (Gale & Taylor, 1947).

Conditions of growth. The organism was grown for 16 hr. at 30° in the 'deficient medium B' consisting of a salt-mixture

solution containing 1% glucose, 0.1% arginine and 0.2% marmite (Gale, 1947). The medium was distributed in 150 ml. quantities in Roux bottles.

Determination of glutamic and aspartic acids. Glutamic acid was determined manometrically by use of the specific glutamic acid decarboxylase preparation (Gale, 1945). Aspartic acid was determined by the manometric method of Krebs (1950) using glutamic acid decarboxylase in conjunction with glutamic-aspartic transaminase. The accumulation of these acids within the cells was studied by the methods previously described (Gale, 1947, 1951*b*).

Determination of combined glutamic acid. For the determination of total glutamic acid, thick suspensions of cells were hydrolysed for 18 hr. in boiling 6*N*-HCl, the acid removed by evaporation to dryness *in vacuo*, the hydrolysate dissolved in water and the pH adjusted to 4.5 before estimation with the decarboxylase preparation. The total glutamic acid was then corrected for the free glutamic acid content to give combined glutamate. Solutions obtained after removal of cells by centrifuging were also examined for combined glutamate in a similar fashion.

Chromatographic investigation of solutions. Incubation mixtures were freed from bacteria by centrifuging and then reduced to one-tenth of their original volume by evaporation under reduced pressure at a temperature below 40°; after neutralization they were deproteinized by the addition of 1 vol. ethanol and 6 vol. CHCl₃, the samples centrifuged and the aqueous layer used for chromatographic examination.

The general procedures used for paper chromatography were those described by Consden, Gordon & Martin (1944).