[8] Waldenström, J., and Wendt, S., *Z. Physiol. Chemie*, **259**, 157 (1939).

[9] Prunty, F. T. G., *Biochem. J.*, **39**, 449 (1945).

[10] Nicholas, R. E. H., and Rimington, C., *Scand. J. Clin. Lab. Investig.*, **1**, 12 (1949).

[11] Chu, T. C., Green, A. A., and Chu, E. J. H., *J. Biol. Chem.*, **190**, 643 (1951).

[12] Falk, J. E., and Benson, A., *Biochem. J.*, **53**, XXXII (1953).

[13] Bogorad, L., and Granick, S. (unpublished data).

[14] Schneider, W. C., chapter in Umbreit, W. W., Burris, R. H., and Stauffer, J. F. S., *Manometric Techniques and Tissue Metabolism*, Burgess Publishing Co., Minneapolis, Minn., 1949, 227 pp.

[15] Gilder, H., and Granick, S., *J. Gen. Physiol.*, **31**, 103 (1947).

[16] Bogorad, L., and Granick, S., *J. Biol. Chem.*, **202**, 793 (1953).

[17] Granick, S., Bogorad, L., and Jaffe, H., *Ibid.*, **202**, 801 (1953).

[18] Corwin, A. H., and Andrews, J. S., *J. Am. Chem. Soc.*, **58**, 1086 (1936).

[19] Corwin, A. H., and Andrews, J. S., *Ibid.*, **59**, 1973 (1937).

[20] London, I. M., and Yamasaki, M., *Fed. Proc.*, **11**, 250 (1952).

[21] Note added in proof. Subsequent to the completion of this manuscript a note appeared in which the synthesis of uroporphyrin, coproporphyrin, and protoporphyrin from porphobilinogen by preparations of hemolyzed chicken erythrocytes was reported. The authors also described the synthesis of mainly coproporphyrin from porphobilinogen by supernatant fractions from hemolyzed erythrocyte preparations centrifuged at high speeds. (Falk, J. E., Dresel, E. I. B., and Rimington, C., *Nature* **172**, 292 (1953).)

---

*COMPETITION AMONG IONIC SPECIES IN CATION ACTIVATION OF ENZYMES**

BY C. E. HAGEN AND P. C. JACKSON

BUREAU OF PLANT INDUSTRY, SOILS, AND AGRICULTURAL ENGINEERING, BELTSVILLE, MARYLAND

Communicated by S. B. Hendricks, September 29, 1953

Activation of many enzymes is ascribed to formation of catalytically active complexes of the enzyme-protein and a specific cation. Since ligands are not highly specific in complexing cations, it is evident that a number of cations may inhibit the enzyme by competing with the activating ions for available binding sites. In many systems, however, the activating cation at higher concentrations causes inactivation of the enzyme.[1, 2] The effect of cation hydrolysis and the enzyme-protein complexes with the resulting ionic species are considered in this paper. An explanation is presented for the distinct cation concentration optimum and its correct position is found without reference to measurement of enzymatic activities.

The equilibrium between manganese and L-leucine aminopeptidase,[3] for example, has been represented as

$$\text{Protein} + \text{Mn}^{++} \rightleftharpoons (\text{Mn}^{++}\cdot\text{protein}), \qquad (1)$$

an apparently self-evident expression which needs to be examined in detail with respect to Mn^{++}. When the products of hydrolysis of a metal are considered, the total concentration would include Mn^{++}, $Mn(OH)_2$, $(MnOH)^+$ and (Mn·protein).

$$[\Sigma Mn] = [Mn^{++}] + [MnOH^+] + [Mn(OH)_2] + [Mn\cdot protein] \quad (2)$$

so that the mathematical expression for equation (1) becomes:

$$(K)\,[Protein]\{[\Sigma Mn] - [MnOH^+] - [Mn(OH)_2] - [Mn\cdot protein]\} = [Mn\cdot protein]. \quad (3)$$

In view of these considerations, an explanation for specific cation activation of enzymes is presented which considers complexing by an enzyme-protein of the ionic species arising from the hydrolysis of the activating cation, and self-competition of the polyvalent cations. The latter proved particularly important.

Derivation and Discussion of Equations.—The mass action expression for the association of a divalent cation, M^{++}, with an enzyme-protein, E, where the binding sites are monovalent and independent, is shown by equation (4); an enzyme with paired monovalent sites is expressed by equation (5)

$$K = \frac{[E_2M]}{[E]^2\,[M]} \quad (4)$$

$$K = \frac{[EM]}{[E]\,[M]} \quad (5)$$

The enzymatic activity in the presence of non-limiting substrate is proportional to the concentration of the enzyme-cation complex, E_2M or EM. According to equation (4), for ideal solutions in which only [E] is limiting, the logarithm of the enzymatic activity, $[E_2M]$, is proportional to the logarithm of the enzyme concentration. With paired monovalent sites, equation (5), the enzymatic activity, [EM], is proportional to the enzyme concentration. Paired monovalent sites are inferred from numerous enzyme concentration curves.[1, 4, 5]

According to equation (5), [EM] would increase with increasing concentrations of cation until the enzyme becomes saturated. Other possibilities must be considered, however, since measurements[1, 2] show that increasing concentrations of the activating cation produce an increase in activity followed by inhibition. The nature of the curves suggests that at increased concentrations, the cations compete for the positions required for the formation of the active complex, EM, by increasing the probability of formation of an inactive complex, $E(M^+)_2$, as described by equation (6)

$$E^{--} + M^{++} \underset{}{\overset{k_1}{\rightleftharpoons}} (EM)^{\pm} \quad (EM)^{\pm} + M^{++} \underset{}{\overset{k_2'}{\rightleftharpoons}} E(M^+)_2. \quad (6)$$

The effective concentration of activating cation is limited by the hydrolysis of the cation, as shown by equations (7) and (8).

$$M^{++} + H_2O \rightleftharpoons (MOH)^+ + H^+ \quad (7)$$

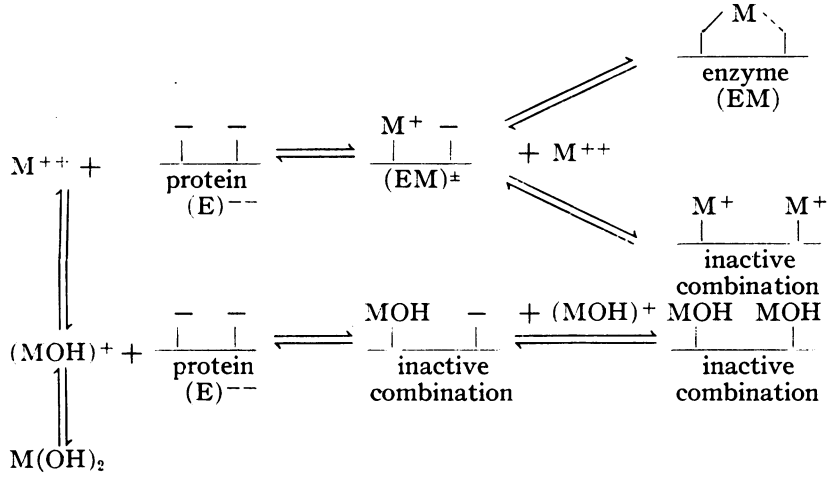
$$(MOH)^+ + H_2O \rightleftharpoons M(OH)_2 + H^+. \quad (8)$$

Formation of $(MOH)^+$ ion introduces the possibility that it, too, is bound by the enzyme site to form a complex, $E(MOH)_2$, equation (9).

$$E^{--} + (MOH)^+ \underset{}{\overset{k_1''}{\rightleftharpoons}} (EMOH)^- \quad (EMOH)^- + (MOH)^+ \underset{}{\overset{k_2''}{\rightleftharpoons}} E(MOH)_2. \quad (9)$$

The uncharged hydroxide, $M(OH)_2$, is not considered to be bound by the enzyme site.

The several reactions are shown in the following scheme:



The enzymatic system at constant temperature, constant pH and non-limited substrate is described by the following equations:

Cation Hydrolysis:

$$K_{1h} = \frac{[MOH^+][H^+]}{[M^{++}]} \quad (10)$$

$$K_{2h} = \frac{[M(OH)_2]\,[H^+]}{[MOH^+]} \quad (11)^\dagger$$

Protein-cation Dissociation:

$$K_{1d} = \frac{[E^{--}]\,[M^{++}]}{[EM]} = \left\{\frac{[E^{--}]\,[M^{++}]}{[EM^\pm]}\right\} \cdot \left\{\frac{[EM^\pm]}{[EM]}\right\} = k_1 k_2 \quad (12)$$

$$K_{2d} = \frac{[E^{--}]\ [M^{++}]^2}{[E(M^+)_2]} = \left\{\frac{[E^{--}]\ [M^{++}]}{[EM^{\pm}]}\right\} \cdot \left\{\frac{[EM^{\pm}]\ [M^{++}]}{[E(M^+)_2]}\right\} = k_1k_2' \tag{13}$$

$$K_{3d} = \frac{[E^{--}]\ [MOH^+]^2}{[E(MOH)_2]} = \left\{\frac{[E^{--}]\ [MOH^+]}{[EMOH^-]}\right\} \cdot \left\{\frac{[EMOH^-]\ [MOH^+]}{[E(MOH)_2]}\right\} = k_1''k_2''. \tag{14}$$

Enzyme Conservation:

$$[E] = [E^{--}] + [EM] + [E(M^+)_2] + [E(MOH)_2]. \tag{15}$$

Cation Conservation:

$$[M] = [M^{++}] + [MOH^+] + [M(OH)_2] + [EM] + 2[E(M^+)_2] + 2[E(MOH)_2]. \tag{16}$$

In equations (10)–(16) there are seven variables; [EM], $[E(M^+)_2]$, $[E(MOH)_2]$, $[MOH^+]$, $[M(OH)_2]$, $[M^{++}]$, and $[E^{--}]$; and three parameters, $[H^+]$, [M] and [E]. These equations may be partially combined and solved by mapping, or all variables except EM, the active complex, eliminated. The latter gives the cubic equation:

$$\alpha(EM)^3 + \beta(EM)^2 + \gamma(EM) - \mu = 0 \tag{17}$$

where α, β, γ, and μ are the following functions of the parameters and the several equilibrium constants:

$$\alpha = 4(K_{1d})^3\{(K_{1h})^2(K_{2d}) + (K_{3d})[H^+]^2\}^2 - (K_{1d})(K_{2d})(K_{3d})[H^+]^2 \times \{(K_{1h})^2(K_{2d}) + (K_{3d})[H^+]^2\}$$

$$\beta = 2(K_{1d})(K_{2d})(K_{3d})[H^+]^2[E]\{(K_{1h})^2(K_{2d}) + (K_{3d})[H^+]^2\} - (K_{2d})^2 \times (K_{3d})^2[H^+]^2\{[H^+]^2 + (K_{1h})[H^+] + (K_{1h})(K_{2h})\} + 4(K_{1d})^2 \times (K_{2d})(K_{3d})\{[H^+]^2 + (K_{1h})[H^+] + (K_{1h})(K_{2h})\}\{(K_{1h})^2(K_{2d}) + (K_{3d})[H^+]^2\}$$

$$\gamma = (K_{1d})(K_{2d})(K_{3d})[H^+]^2[M]\{[M] - 2[E]\}\{(K_{1h})^2(K_{2d}) + (K_{3d}) \times [H^+]^2\} + (K_{2d})^2(K_{3d})^2[H^+]^2\{[M] + [E]\}\{[H^+]^2 + (K_{1h})[H^+] + (K_{1h})(K_{2h})\} + (K_{1d})(K_{2d})^2(K_{3d})^2\{[H^+]^2 + (K_{1h})[H^+] + (K_{1h}) \times (K_{2h})\}$$

$$\mu = (K_{2d})^2(K_{3d})^2[H^+]^2[E][M]\{[H^+]^2 + (K_{1h})[H^+] + (K_{1h})(K_{2h})\}.$$

Equation (17) shows that when [E] and $[H^+]$ are maintained constant, [EM], the concentration of the active enzyme, increases to a maximum and then decreases as the cation concentration is increased. One should note that $[E(M^+)_2]$ and $[E(MOH)_2]$ continue to increase and the sum of the two asymptotically approaches complete enzyme saturation. Since the coefficients α and β are not functions of [M], they are constant when

[E] and [H^+] are maintained constant. Initially, as cation concentration is increased, [EM] increases because μ varies directly with [M] and the terms in γ containing [M] and $[M]^2$ are insignificant. With further increases of cation, the [M] term in γ is significant and [EM] becomes constant. This describes the optimal concentration of EM. With even greater increases of cation, the $[M]^2$ term becomes significant and the result is reduced concentrations of EM. The location of the peak of maximum [EM] value is determined by the dissociation and hydrolysis constants, with the sharpness of the peak depending on the relative values of the dissociation constants.

In the expressions for α, β, and γ the $(K_{1h})^2(K_{2d})$ term in equation (17) reflects the effect of the formation of $E(MOH)_2$, and the $(K_{3d})[H^+]^2$ term, the $E(M^+)_2$ complex. Thus, if $(K_{1h})^2(K_{2d})$ is insignificant in comparison to $(K_{3d})[H^+]^2$, inhibition of EM due to formation of $E(MOH)_2$ is negligible, and the inhibition is due to formation of $E(M^+)_2$.

TABLE 1

HYDROLYSIS AND DISSOCIATION CONSTANTS AT 25°C.

CONSTANT[a]	DIVALENT METAL		
	MANGANESE	MAGNESIUM	COBALT
K_1 hydrolysis	1×10^{-13}[b]	5×10^{-12}	1×10^{-12}
K solubility product	5×10^{-14}	9×10^{-12}	3×10^{-15}
K_2 hydrolysis (calc.)	1×10^{-2}	5×10^{-6}	1×10^{-2}
K_1 dissociation	1×10^{-5}	5×10^{-4}	1×10^{-6}
K_2 and K_3 dissociation	1×10^{-7}	5×10^{-6}	1×10^{-9}

[a] All constants were rounded out to the nearest half power.

[b] The K_{1h} of cobalt, nickel, and zinc change by two factors of ten from 100° to 25°C. The same extrapolation was applied to approximate the K_{1h} of manganese at 25°C. The K_{1h} of manganese at 100°C. is 2×10^{-11}.[7]

Whenever the terms $(K_{1h})[H^+]$ and $(K_{1h})(K_{2h})$ are insignificant compared to $[H^+]^2$, and $(K_{1h})^2(K_{2d})$, as compared to $(K_{3d})[H^+]^2$, the enzymatic activity is independent of the hydrogen-ion concentration. This condition may hold only in a limited region. The general dependence of cation activities on [H^+] is given by equation (17), but the consequences of this dependence on [H^+], as a variable influencing enzymatic activity, requires consideration of enzyme hydrolysis which is not considered here.

Hydrolysis and Dissociation Constants.—The K_{1h} for each metal, table 1, is an average of several values obtained from literature.[6-8] Where the value of K_{2h} was not available, an apparent constant was calculated, and designated as such, from the solubility product as follows:

$$K_{2h} = \frac{(K_w)^2[\text{soluble } M(OH)_2]}{(K_{sp})(K_{1h})}. \qquad (18)$$

Since the solubility of the undissociated metal hydroxides has not been determined, it was assumed that these substances are completely ionized.

From previous considerations, the enzyme-protein has been inferred to have paired monovalent sites, with an imposed limitation on both positions of equal association values. From statistical and electrostatic considerations, however, the relative stability of the metal complex with the first position would be expected to be greater than that with the second. An internally unbiased scheme has been devised for an approximation of these constants. A number of dissociation constants for each metal with organic acids, amino acids, and peptides were calculated from an average of chelate stability constants.[9] In each case, the dissociation constant of the enzyme-metal complex, K_{1d}, K_{2d}, and K_{3d}, was assumed to be a factor

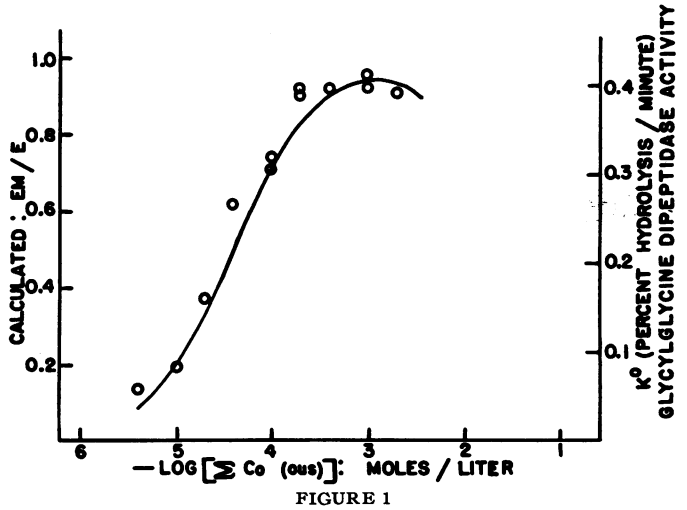


FIGURE 1

Activation of glycylglycine dipeptidase by cobalt, at pH 7.8. The solid line describes the curve derived from equation (17). The points are taken from the experiments of Smith.[10]

of ten smaller than this average, since it appears likely that the dissociation of a protein-metal complex would be smaller than these averages. Only orders of magnitude are important.

Since the two positions of the paired monovalent site exist in the same molecule, they do not have independent freedom for orientation with respect to the divalent cation. When the first position is occupied by a divalent cation, the position of the second limits bonding to the same cation and the relative value of k_2 is greatly increased. The dissociation constant of EM, K_{1d}, in this treatment, is considered to be that of the first position, k_{1d}.

Estimation of the stability of $E(MOH)_2$ relative to $E(M^+)_2$ necessitates consideration of the effect of hydrogen bonding. If the juxtaposition of

groups which can form hydrogen bridges is unfavorable, or are blanked out by interformation of hydrogen bonding between the ketoimino linkage and amino and carboxyl side chains, no difference in stability would be expected between the two complexes. From these, and from entropy considerations, the simplifying assumption has been made that K_{2d} and K_{3d} for the enzymes have the same order of magnitude, table 1. A different situation is met in cation binding by clays where $K_{2d} > K_{3d}$ which enhances formation of the complex with the basic ion.

Agreement with Experimental Observations.—Agreement between observed enzymatic activities and values calculated from equation (17) are shown in figures 1, 2, and 3, at various metal concentrations. In figure 1, and curve (*a*) of figure 2, further increases of metal would cause the theoretical curve to descend in the manner shown by figure 3 and curve (*b*) of figure 2; experimental results, however, were not available beyond the metal concentration shown. The change in pH from 7.6 to 7.8, figure 2, translocates the curve to greater manganese concentrations. Since higher pH values decrease $[M^{++}]$ relative to the concentrations of $(MOH)^+$ and $M(OH)_2$, greater total metal concentration is required to produce the same effect as at lower pH values. In all of the cases studied, $[MOH^+]$ is insignificant as compared to $[M^{++}]$ up to pH 10; consequently, unless the enzyme has an affinity for $(MOH)^+$ greater by several factors of ten (6 or greater, at pH 8, in most cases) than it has for M^{++}, the inactivation is caused by formation of $E(M^+)_2$. Although additional binding may be exerted by groups of the protein moiety on the metal, it apparently is not limiting. Enzyme concentrations affect only the absolute concentrations of the metal-enzyme complexes but not their concentrations relative to each other. With the values of the constants given, the slopes of the metal concentration curves would change only at enzyme concentrations greater than 10^{-5} *M*. Therefore, concentrations of 10^{-8} *M* were used for calculations. The agreement of equation (17) with experimental results indicates that the effect of buffers is insignificant in the systems studied, as was expected from their probable dissociation constants. The theoretical metal concentration curves agree, so far as tested, with experimental results on enzymes other than the examples presented.

The assumption that EM is the active complex is supported by the fact that $[E(MOH)_2]$ or $[E(M^+)_2]$ never decreases with increasing [M]. If the $(MOH)^+$ affinity for the enzyme is greater than that of M^{++}, then it must be greater by a factor of 10^6, which is unlikely, in order to describe the experimental results. In addition, experimental results do not support the likelihood that the dissociation constant for EM is equal to, or smaller than, the dissociation constant for $E(MOH)_2$ or $E(M^+)_2$.

Although the system presented does not include all the possible inter-

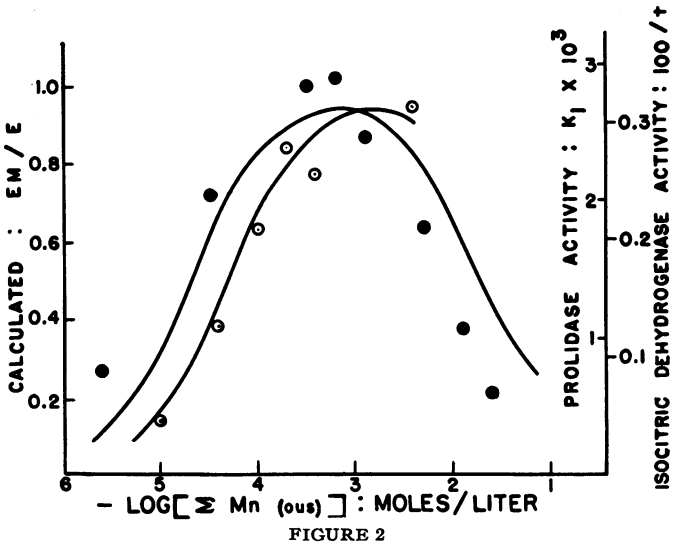


FIGURE 2

Activation by manganese of prolidase, at pH 7.8 (curve (a), open circles), from Adams, *et al.*,[4] and of isocitric dehydrogenase, at pH 7.6 (curve (b), solid circles), from Adler, *et al.*[1] The solid lines describe the respective curves derived from equation (17).

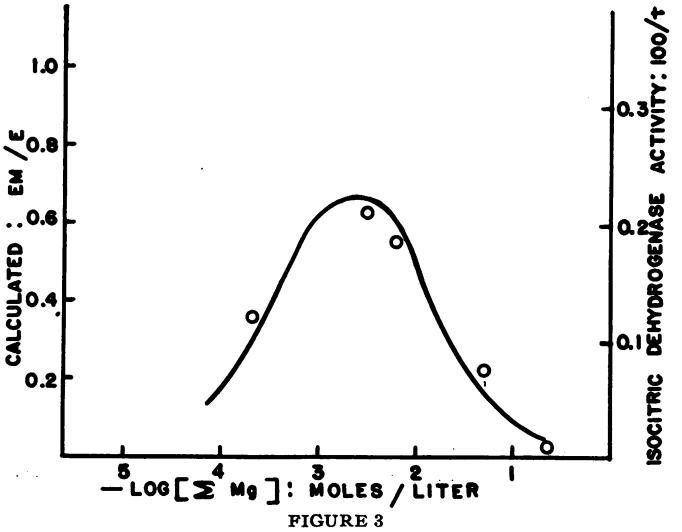


FIGURE 3

Activation of isocitric dehydrogenase by magnesium, at pH 7.6. The solid line describes the curve derived from equation (17). The points are taken from the experiments of Adler, *et al.*[1]

actions of the components, the agreement with experimental results indicates that it describes the mechanism for cation activation of enzymes.

* This investigation was supported in part by the U. S. Atomic Energy Commission.

† Although the active concentration of $M(OH)_2$ in the solid phase is generally assumed to be constant, as the entire mass per unit volume does not participate in a reaction, this treatment has admitted the $M(OH)_2$ as a variable in the equation.

[1] Adler, E., v. Euler, H., Gunther, G., and Plass, M., *Biochem. J.*, **33**, 1028 (1939).

[2] Malstrom, B. G., *Nature*, **171**, 392 (1953).

[3] Smith, E. L., *J. Biol. Chem.*, **163**, 15 (1946).

[4] Adams, E., and Smith, E. L., *Ibid.*, **198**, 671 (1952).

[5] Ochoa, S., in Sumner and Myrback, *The Enzymes*, Academic Press, New York, 1951, p. 1220.

[6] Gayer, K. H., and Garrett, A. B., *J. Am. Chem. Soc.*, **74**, 1436 (1952).

[7] Kullgren, C., *Z. Physik. Chem.*, **85**, 466 (1913).

[8] Stock, D. I., and Davies, C. W., *Trans. Far. Soc.*, **44**, 856 (1948).

[9] Martell, A. E., and Calvin, M., *Chemistry of the Metal Chelate Compounds*, Prentice-Hall, New York, 1952, p. 514.

[10] Smith, E. L., *J. Biol. Chem.*, **173**, 571 (1948).

---

*ENZYMATIC REACTIONS IN PURINE DECOMPOSITION BY PREPARATIONS OF CLOSTRIDIUM ACIDI-URICI**

BY NORMAN S. RADIN† AND H. A. BARKER

DEPARTMENT OF PLANT BIOCHEMISTRY, UNIVERSITY OF CALIFORNIA, BERKELEY

Communicated October 26, 1953

Clostridium acidi-urici ferments several purines including uric acid, xanthine, and guanine.[1, 2] The products of these fermentations are mainly acetic acid, carbon dioxide, and ammonia. Experiments with cell suspensions and C^{14}-labeled uric acid have established the source of some of the carbon atoms in the products.[3] Also various types of evidence have been obtained which indicate that glycine may be an intermediate in the purine fermentation. Glycine is readily decomposed by cell suspensions when a fermentable purine is simultaneously supplied, and it is formed from uric acid by a physiologically similar bacterium, *C. cylindrosporum*.[2] These and other lines of evidence indicate that the mechanism of purine decomposition by these bacteria is somewhat similar to the mechanism of purine synthesis in animals.[4]

Up to now all experiments on the purine fermentation have been done with intact cells. This report describes the anaerobic conversion of xanthine to glycine, ammonia, carbon dioxide, and formate by dried cell suspensions and cell-free extracts of *C. acidi-urici*. The enzymatic decomposition of glycine, serine, pyruvate, and formate by extracts is also reported.