[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF WISCONSIN]

Some Mechanisms for the Interpretation of the Effects of *p*H and Buffer Salts on a Simple Enzymatic Reaction

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Equations for the initial reaction rate for enzyme mechanisms involving interaction between the enzyme and hydrogen ions and buffer ions have been obtained by the steady state method. Provision is made for the ionization of two acid groups in the enzyme which affect the catalytic activity. The variation of maximum initial velocities, Michaelis constants and competitive inhibition constants with *p*H and buffer concentrations are discussed. These rate equations are in agreement with the experimental results for the fumarase reaction. The inclusion of buffer and hydrogen ion equilibria in mechanisms leads to an altered interpretation of kinetic and inhibition constants and emphasizes the effects of bound buffer and inhibitor upon the ionization of groups affecting the catalytic activity. For example, the values of competitive inhibition constants for a series of compounds at a certain *p*H may not be in the order of the equilibrium constants for the combination of the inhibitors with a given ionized form of the enzyme. However, such equilibrium constants and the shifts of ionization constants for the enzyme may be obtained from an investigation of the effect of *p*H upon inhibition. The Haldane relation also applies in the case of these more complicated mechanisms.

The mechanisms to be described have been devised as an aid to the interpretation of over-all kinetic and inhibition data for fumarase. However, the equations and method of approach should have considerably wider applicability than to this one enzyme. Although the initial velocities of the forward and reverse reactions catalyzed by fumarase follow the equation of Michaelis and Menten[1] at sufficiently low substrate concentrations, the Michaelis constants and maximum initial velocities vary considerably with the nature, concentration and *p*H of the buffer.[2] Mechanisms involving buffer–enzyme interactions which lead to a dependence of Michaelis constants, maximum initial velocities and inhibition constants on buffer concentration have been discussed by Alberty and Bock,[3] and it is the purpose of this article to extend this treatment to include the effect of hydrogen ions. The effect of hydrogen ions on the rate of an enzymatic reaction was interpreted by Michaelis and co-workers[4,5] as being due to the ionization of groups in the enzyme molecule. This hypothesis has been utilized by a number of investigators[6–13] who have interpreted their kinetic data in terms of ionization of the enzyme or enzyme–substrate complexes. The effects of various ions on the ionization constants of essential groups in fumarase have been discussed qualitatively by Massey.[14] Recently, Waley[15] has derived a rate equation allowing for two ionizable groups on the enzyme and on the enzyme–substrate complex, Dixon[16] has discussed the determination of an indefinite number of ionization constants of enzyme and enzyme–substrate or enzyme–inhibitor complexes from the *p*H variation of the Michaelis constant or inhibition constant, and Botts and Morales[17] have discussed the effect of one equilibrium with a rate modifying substance which may be either a hydrogen ion or another component of buffer.

In order to simplify the present treatment, ionization of substrate or inhibitor will not be introduced although this could be readily included.[6,15,18] Only the effects of various equilibria will be considered, and ionic strength effects such as those discussed by Kistiakowsky and Shaw[19] are perforce omitted, although they will be superimposed upon the effects of equilibria.

*p*H Effects in the Absence of Buffer Effects

As pointed out by Michaelis and co-workers[4,5] it is necessary to postulate two types of ionizable groups affecting the enzymatic activity in order to account for a reversible loss in activity in both acidic and basic solutions. The simplest way in which this can be formulated while retaining a mechanism involving an enzyme–substrate complex is

$$\begin{array}{ccccc} E^{n-1} & \underset{}{\overset{K_{aE}}{\rightleftharpoons}} & E^{n} & \underset{}{\overset{K_{bE}}{\rightleftharpoons}} & E^{n+1} \\ & & K_{ES}\updownarrow & & \\ E^{n-1}S & \underset{}{\overset{K_{aES}}{\rightleftharpoons}} & E^{n}S & \underset{}{\overset{K_{bES}}{\rightleftharpoons}} & E^{n+1}S \\ & & k_1\downarrow & & \\ & & E^{n} + P & & \end{array} \qquad (I)$$

where n is the net number of negative charges on groups in the neighborhood of the active site. The charge on the active form of the enzyme is represented by the non-committal n. The two groups which affect the catalytic activity will be referred to as a and b since they are responsible for

(1) L. Michaelis and M. L. Menten, *Biochem. Z.*, **49**, 333 (1913).
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(17) J. Botts and M. Morales, *Trans. Faraday Soc.*, **49**, 696 (1953).
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the acidic and basic regions of the pH–maximum initial velocity curve, and K_a and K_b are acid dissociation constants. The fact that the equilibria $E^{n-1}S + S \rightleftarrows E^{n-1}S$ and $E^{n+1} + S \rightleftarrows E^{n+1}S$ are not indicated is not because they are assumed to be lacking but because they are not independent of the other reactions.

The equation for the initial rate of a reaction following a mechanism such as I may be derived assuming either that the equilibria are adjusted rapidly[1] in comparison with the rate of breakdown of E^nS or that the various complexes are in a steady state[20] (that is, $d(E^nS)/dt = 0$, $d(E^{n-1})/dt = 0$, etc.). In the case of mechanisms discussed in this article the same rate equations are obtained by both methods. The difference is that the steady state derivations show that the Michaelis constants for complexes which break down to yield product are not equilibrium constants but of the type shown by Briggs and Haldane.[20] In order to simplify the nomenclature for the discussion of mechanisms in this paper each reversible step is represented by a capital K which is an equilibrium constant if the complex indicated in the subscript does not dissociate to form product but is of the Briggs–Haldane type when the complex does yield product. Lower case k's are used to designate the pH-independent first-order rate constants for the formation of product.

The steady state treatment of mechanism I has previously been given by Waley,[15] who assumed that the hydrogen ion equilibria are adjusted rapidly. For the case that $(S) \gg (E)_0$, where $(E)_0$ is the total molar concentration of enzymatic sites, the initial reaction velocitity (v) is given by the usual Michaelis[1] equation.

$$v = \frac{V}{1 + K_m/(S)} \qquad (1)$$

where

$$V = \frac{k_1(E)_0}{1 + (H^+)/K_{aES} + K_{bES}/(H^+)} \qquad (2)$$

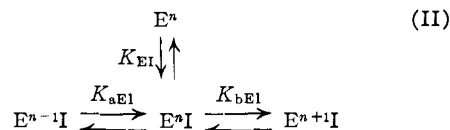
$$K_m = K_{ES}\frac{1 + (H^+)/K_{aE} + K_{bE}/(H^+)}{1 + (H^+)/K_{aES} + K_{bES}/(H^+)} \qquad (3)$$

Expression of the result of the steady state treatment in the form of equation 1 has the advantage that V and K_m are the constants which are usually obtained from experimental data as by use of a Lineweaver–Burk[21] plot. Equation 3 is equivalent to that derived by Dixon[16] who has discussed rules for the determination of the various ionization constants. According to equation 2 a plot of V *vs.* pH should have a symmetrical bell shape determined by the ionization constants for the enzyme–substrate complex. A method for the calculation of these constants from the shape of the plot has been developed and used by Alberty and Massey.[22,23] If the four pK values which determine the pH variation of K_m are rather closely spaced it may be extremely difficult to separate them by use of equation 3, but pK_{aE} and pK_{bE} may be obtained from a plot of V/K_m (the reciprocal of the slope of a Lineweaver–Burk plot) *vs.* pH by the same method used in the case of V.[22] For a reversible enzymatic

$$\frac{V}{K_m} = \frac{k_1(E)_0/K_{ES}}{1 + (H^+)/K_{aE} + K_{bE}/(H^+)} \qquad (4)$$

reaction it is to be expected that the same values for K_{aE} and K_{bE} will be obtained for the forward and reverse reactions while K_{aES} and K_{bES} may be different for the substrates for the forward and reverse reactions.

Competitive Inhibition.—If a competitive inhibitor is present it is necessary to add the following to the preceding mechanism



The rate equation obtained by a steady-state treatment is

$$v = \frac{V}{1 + K_m(1 + (I)/K_I)/(S)} \qquad (5)$$

where K_I, the competitive inhibition constant, is

$$K_I = K_{EI}\frac{1 + (H^+)/K_{aE} + K_{bE}/(H^+)}{1 + (H^+)/K_{aEI} + K_{bEI}/(H^+)} \qquad (6)$$

Equation 6 is in agreement with the discussion by Dixon[16] of the effect of pH on competitive inhibition constants. If the various pK values which determine the pH variation of K_I are not well separated, the calculation of the various pK's may be facilitated by plotting $K_I V/K_m$ *vs.* pH since a symmetrical bell-shaped plot depending upon pK_{aEI} and pK_{bEI} should be obtained.

In studying the effect of the structure of inhibitors upon their affinity for the enzyme the value of K_{EI} for the reaction of inhibitor with a certain ionic form of the enzyme should be more significant than the pH-dependent K_I value. The relative values of K_I for a series of compounds at a given pH may not give a reliable indication of the relative values of K_{EI} because of differences in the shifts of the ionization constants of groups in the enzyme produced by the inhibitors. For example, if two inhibitors with identical values of K_{EI} are tested at a pH in the region between pK_{aE} and pK_{aEI}, the inhibitor which produces the greater increase in pK_{aEI} will be the better inhibitor.

pH Effects in the Presence of Buffer Effects

Mechanism I does not provide for the effect of buffer upon the kinetic constants. In the case of fumarase the Michaelis constants, maximum initial velocities and competitive inhibition constants are very dependent upon the concentration of phosphate buffer.[2] The increase in V with phosphate concentration indicates an activation resulting from combination of phosphate ions at non-enzymatic sites, and the fact that K_m and K_I continue to increase with buffer concentration even after V has become constant indicates an inhibition resulting from combination with the enzymatic site. The activating effect of a component of the buffer (B) may be represented as

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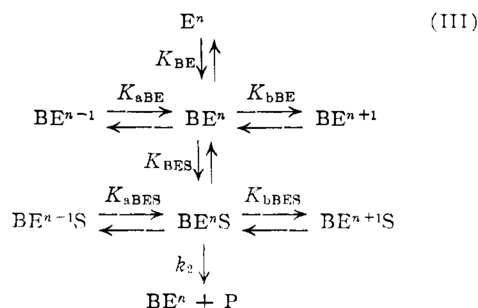
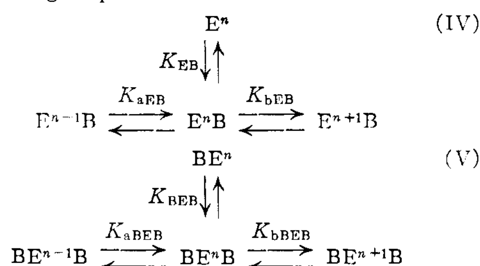


The combination of B with a neighboring site at which it affects the kinetic constants is represented by writing B to the left of E. If, in addition, B competes with S for the enzymatically-active site, the following steps must be added.



The initial velocity for a reaction following the mechanism given by I, III, IV and V is given by the usual Michaelis equation 1. The expressions for V and K_m may be arranged in two ways depending upon whether it is desired to emphasize their variation with (B) or pH.

$$K_a'' = \frac{1 + (B)(1/K_{BE} + 1/K_{EB}) + (B)^2/K_{BE}K_{BEB}}{1/K_{aE} + (B)(1/K_{BE}K_{aBE} + 1/K_{EB}K_{aEB}) + (B)^2/K_{BE}K_{BEB}K_{aBEB}} \quad (16)$$

$$K_b'' = \frac{K_{bE} + (B)(K_{bBE}/K_{BE} + K_{bEB}/K_{EB}) + (B)^2K_{bBEB}/K_{BE}K_{BEB}}{1 + (B)(1/K_{BE} + 1/K_{EB}) + (B)^2/K_{BE}K_{BEB}} \quad (17)$$

If the buffer concentration is held constant and the pH varied

$$V = \frac{V'}{1 + (H^+)/K_a' + K_b'/(H^+)} \quad (7)$$

where

$$V' = k_1(E)_0 \frac{1 + k_2K_{ES}(B)/k_1K_{BE}K_{BES}}{1 + K_{ES}(B)/K_{BE}K_{BES}} \quad (8)$$

$$K_a' = \frac{1 + K_{ES}(B)/K_{BE}K_{BES}}{1/K_{aES} + K_{ES}(B)/K_{BE}K_{BES}K_{aBES}} \quad (9)$$

$$K_b' = \frac{K_{bES} + K_{bBES}K_{ES}(B)/K_{BE}K_{BES}}{1 + K_{ES}(B)/K_{BE}K_{BES}} \quad (10)$$

Thus the maximum initial velocity will vary with pH in the same manner as for mechanism I, but the three parameters V', K_a' and K_b' may vary with the buffer concentration. The equilibrium constants for steps IV and V do not enter the expression for V since at infinite substrate concentration B is completely displaced from the enzymatic site by substrate.

If terms involving (B) in equation 7 are brought together, the maximum initial velocity may be written as

$$V = \frac{V_0K_B + V_B(B)}{K_B + (B)} \quad (11)$$

where V_0 is given by equation 2 and

$$V_B = \frac{k_2(E)_0}{1 + (H^+)/K_{aBES} + K_{bBES}/(H^+)} \quad (12)$$

$$K_B = \frac{K_{BE}K_{BES}(1 + (H^+)/K_{aES} + K_{bES}/(H^+))}{K_{ES}(1 + (H^+)/K_{aBES} + K_{bBES}/(H^+))} \quad (13)$$

Equation 11 is identical with the empirical equation found to represent the data for fumarase in phosphate buffer. It will be noted that K_B may have different values for the forward and reverse reactions.

The maximum initial velocity V at a particular pH will increase with the buffer concentration if $V_B > V_0$. For this to be the case it is not necessary that $k_2 > k_1$. The effect of the bound buffer ions on the ionization constants of the two essential groups in the enzyme may be the determining factor. In the case of fumarase Massey[14] has shown that the pH of optimal activity is different for various buffers. Thus increasing the concentration of a certain buffer may cause V to increase at one pH but decrease at another.

The variation of the Michaelis constant with pH for the mechanism represented by I, III, IV and V is given by

$$K_m = K_m' \frac{1 + (H^+)/K_a'' + K_b''/(H^+)}{1 + (H^+)/K_a' + K_b'/(H^+)} \quad (14)$$

where K_a' and K_b' are given by equations 9 and 10 and

$$K_m' = K_{ES} \frac{1 + (B)(1/K_{BE} + 1/K_{EB}) + (B)^2/K_{BE}K_{BEB}}{1 + K_{ES}(B)/K_{BE}K_{BES}} \quad (15)$$

Thus at a given buffer concentration the Michaelis constant will vary with pH in the same manner as if there were no buffer effect (*cf.* equation 3), but the various constants in equation 14 are functions of the buffer concentration. The apparent ionization constants K_a'' and K_b'' may be obtained from experimental data by use of a plot of V/K_m.

The variation of the Michaelis constant with buffer concentration at constant pH is given by

$$K_m = K_{mo} \frac{1 + L_1(B) + L_2(B)^2}{1 + (B)/K_B} \quad (18)$$

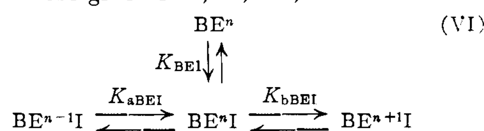
where K_{mo} is given by equation 3, K_B by equation 13 and

$$L_1 = \frac{(1 + (H^+)/K_{aBE} + K_{bBE}/(H^+))/K_{BE} + (1 + (H^+)/K_{aEB} + K_{bEB}/(H^+))/K_{EB}}{(1 + (H^+)/K_{aE} + K_{bE}/(H^+))} \quad (19)$$

$$L_2 = \frac{(1 + (H^+)/K_{aBEB} + K_{bBEB}/(H^+))}{K_{BEB}K_{BE}(1 + (H^+)/K_{aE} + K_{bE}/(H^+))} \quad (20)$$

Thus K_m may continue to increase with buffer concentration even after V has become constant, and at high buffer concentrations K_m is a linear function of (B).

Competitive Inhibition.—In the case of an inhibitor I which combines only at the enzymatically-active site it is necessary to add the following equilibria to those given in I, II, III, IV and V.



The resulting rate equation is given by 5 with V given by 7 or 11, and K_m given by 14 or 18. The

variation of K_I with *p*H is of the same type as for the simpler mechanism (equation 6) except that the parameters in the following equation may depend upon the buffer concentration.

$$K_I = K_I' \frac{1 + (H^+)/K_a'' + K_b''/(H^+)}{1 + (H^+)/K_a''' + K_b'''/(H^+)} \quad (21)$$

where K_a'' and K_b'' are given by equations 16 and 17, and

$$K_I' = K_{EI} \frac{1 + (B)(1/K_{BE} + 1/K_{EB}) + (B)^2/K_{BE}K_{BEB}}{1 + K_{EI}(B)/K_{BE}K_{BEI}} \quad (22)$$

$$K_a''' = \frac{1 + K_{EI}(B)/K_{BE}K_{BEI}}{1/K_{aEI} + K_{EI}(B)/K_{BE}K_{BEI}K_{aBEI}} \quad (23)$$

$$K_b''' = \frac{K_{bEI} + (B)K_{EI}K_{bBEI}/K_{BE}K_{BEI}}{1 + (B)K_{EI}/K_{BE}K_{BEI}} \quad (24)$$

The variation of the competitive inhibition constant with buffer concentration at constant *p*H is given by

$$K_I = K_{I0} \frac{1 + L_1(B) + L_2(B)^2}{1 + (B)/K_B'} \quad (25)$$

where K_{I0} is given by equation 6, L_1 by equation 19, L_2 by equation 20 and

$$K_B' = \frac{K_{BE}K_{BEI}(1 + (H^+)/K_{aEI} + K_{bEI}/(H^+))}{K_{EI}(1 + (H^+)/K_{aBEI} + K_{bBEI}/(H^+))} \quad (26)$$

It may be noted that K_B is the same as K_B' given by equation 13 except that I is substituted for S.

In the case of poor inhibitors which are of necessity tested at a rather high concentration it is to be expected that the inhibitor may combine with the enzyme at sites other than the enzymatically-active ones. Analysis[3] of equations for the case that I combines at a neighboring site at which it is not a total inhibitor shows that types of inhibition which do not fit into the ordinary classifications may be obtained although v^{-1} remains a linear function of $(S)^{-1}$.

Discussion

It is interesting that rate expressions derived from simple mechanisms may be applicable to more complicated mechanisms. For example, the original treatment by Michaelis and Menten[1] produced the same type of rate equation as the latter steady-state treatment of Briggs and Haldane[20] which led to a different interpretation of K_m. Furthermore, even when buffer[8] or hydrogen ion[15] equilibria, or both together, are added to the Michaelis mechanism, the same familiar rate equation is obtained for a given set of conditions although the interpretation of the maximum initial velocity, Michaelis constant and inhibition constants are further altered. As a further example, the relationship between the equilibrium constant for an over-all reaction of the type F $\rightleftarrows$ M and the Michaelis constants and maximum initial velocities for the forward and reverse reactions originally derived by Haldane[24] for a very simple mechanism has been found to be obeyed by the fumarase reaction for which the various kinetic constants depend upon buffer concentration and *p*H. The Haldane relation

$$K_{eq} = \frac{(M)}{(F)} = \frac{V_F K_M}{V_M K_F} \quad (27)$$

may also be derived from the present mechanisms involving buffer ions and hydrogen ions. It may be readily ascertained that the ratio of V (equation 7) to K_m (equation 14) is independent of K_a' and K_b', and that K_a'' and K_b'' cancel in taking the ratio of V/K_m for the forward reaction to that for the reverse reaction. Similarly, the equations corresponding to equation 27 for more complicated mechanisms[25] are also valid even if the various kinetic constants are dependent upon *p*H and buffer concentration.

Since the reactions of hydrogen ion, buffer and substrate (or competitive inhibitor) with the enzyme are interdependent they are linked functions according to the terminology of Wyman.[26] In general the buffer-linked acid groups and substrate-linked acid groups may be different, but in the present mechanisms it has been assumed that they are the same. In the study of linkage through kinetics measurements it must be remembered that the apparent equilibrium constants for complexes which break down to yield product will be of the type derived by Briggs and Haldane.[20]

It is worth considering what shifts in the ionization constants of groups in the enzyme may result from the binding of S, B and I. If these substances are ions their electrostatic effects on the ionization of the ionizable groups may be predicted. A bound negative ion will be expected to produce an acid-weakening effect ($K_{aE} > K_{aES}$ and $K_{bE} > K_{bES}$). Therefore, in the case of anionic substrates and inhibitors it would be expected that K_m and K_I would increase with increasing *p*H while the opposite would be expected for cationic substrates and inhibitors. A quantitative method for calculating the effect of a charged group on the acidity of a neighboring group was first developed by Bjerrum[27] and has been extended and improved by Kirkwood and Westheimer.[28] The calculated[29] values for the charge separation in dipolar ions and dibasic acids are in good agreement with the free rotation values.

The equations in this article have been based upon the assumption that the values of pK_a and pK_b are constants independent of *p*H. However, this can be only approximately true for real protein molecules since the change of charge of the whole molecule with *p*H and salt concentration has an effect on the ionization of all groups in the molecule. This effect is taken into account in the interpretation of the titration of proteins by assuming that the charge is uniformly distributed over a sphere of the proper size.[30,31]

Buffers such as phosphate present an additional complication since the ratio of the concentrations of monovalent and divalent ions changes with the *p*H. Since it is to be expected that these two ions

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(27) N. Bjerrum, *Z. physik. Chem.*, **106**, 219 (1923).

(28) J. G. Kirkwood and F. H. Westheimer, *J. Chem. Phys.*, **6**, 506, 513 (1938).

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(30) K. Linderstrøm-Lang, *Compt. rend. trav. lab. Carlsberg*, **15**, No. 7 (1924).

(31) G. Scatchard, *Ann. N. Y. Acad. Sci.*, **51**, 660 (1949).

will have different effects upon the enzyme, the mechanism indicated by I, III, IV and V must be further enlarged. This leads to an expression for V which cannot be arranged in the form of 7. Thus a plot of V *vs.* pH may be asymmetrical if the pK of a buffer such as phosphate is in the same region as the activity curve. However, if the buffer concentration is high and one form has a sufficiently great affinity to displace the other, the V–pH curve may be symmetrical and the pK values would correspond to the enzyme saturated with the more strongly bound form of the buffer. Buffers of the uncharged-acid or uncharged-base types have the advantage over phosphate that the concentration of the ionized form of the buffer may be held constant over a wide range of pH.

Since enzymes are proteins it is to be expected, in general, that their properties will depend on the concentration and nature of salts in the solution and upon the pH. Although these effects may be too complicated to express by such simple mechanisms as those used here, the fact that the present equations are in good agreement with the results for fumarase is encouraging.

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NOTES

A Synthesis of 1-C[14]-Labeled Diethyl Ether

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In the course of other work in this Laboratory, need arose for a sample of C[14]-labeled diethyl ether. A search of the literature revealed that, although many preparations of ethyl ether are described,[1] no synthesis of the C[14]-labeled compound could be found. Furthermore, no high yield procedure for small amounts (10–12 g. of product) which could be used directly for this purpose, came to light. It became imperative, therefore, for us to develop a satisfactory process for our own use.

Since it was necessary to label only one ethyl radical, it was decided to use the Williamson synthesis[2] as the basic reaction because it offered maximum economy of radioactive starting material. The procedure ultimately adopted as satisfactory for small batches was based on the experiments of Hunt[3] and of Beilstein[4] on ethyl iodide preparation, and those of Bishop[5] on the reaction of ethyl iodide with sodium ethoxide.[6]

From 11–12 g. of 95% ethanol-1-C[14] (100 microcuries) 32.4 g. (87.6%) of ethyl-1-C[14]-iodide boiling at 70–73° was obtained. Treatment of 26.4 g. of this material with sodium ethoxide gave 11.5 g. (92%) of 1-C[14]-ethyl ether boiling at 33.5–34°.

(1) T. Saussune, *Ann. chim.*, [1] **89**, 273 (1814); J. L. Gay-Lussac, *ibid.*, [1] **95**, 311 (1815); Dumas and Boullay, *ibid.*, [2] **36**, 294 (1827); A. W. Williamson, *ibid.*, [3] **40**, 98 (1854); *Ann.*, **77**, 37 (1851); *ibid.*, **81**, 73 (1852); E. Erlenmeyer, *ibid.*, **162**, 380 (1872); A. W. Titherley, *J. Chem. Soc.*, **79**, 392 (1901).

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(6) The complete experimental details of this preparation have been deposited as Document number 4193 with the ADI Auxiliary Publications Project, Photoduplication Service, Library of Congress, Washington 25, D. C. A copy may be secured by citing the Document number and by remitting $1.25 for photoprints, or $1.25 for 35 mm. microfilm. Advance payment is required. Make checks or money orders payable to: Chief, Photoduplication Service, Library of Congress.

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SAINT PAUL 1, MINNESOTA

N-Cycloalkyl- and N,N-Polymethylenesulfamic Acids

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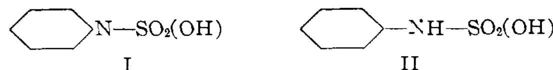
Since considerable quantities of hexa-, hepta- and octamethylenimine were available, it seemed desirable to prepare a few N,N-polymethylenesulfamic acids. These compounds are of interest since they are related to cycloalkylsulfamic acids. The sodium and calcium salt of N-cyclohexylsulfamic acid (Sucaryl or Cyclamate sodium or calcium) are important sweetening agents.[1] It is obvious that N,N-pentamethylenesulfamic acid (I) represents N-cyclohexylsulfamic acid (II) in which the nitrogen atom has been made a part of the ring structure.



The N,N-polymethylenesulfamic acids were obtained by interaction of chlorosulfonic acid with pyrrolidine, hexamethylen-,[2] heptamethylen-[2] or octamethylenimine[2] by the general method used by Audrieth and Sveda[3] for the synthesis of N-cyclohexylsulfamic acid.

No sweet taste could be detected when the solid sodium salts of the N,N-polymethylenesulfamic acids were tested. Furthermore, no sweet taste

(1) *Ind. Eng. News*, **45**, No. 10, 11 (1953).

(2) L. Ruzicka, M. Kobelt, O. Häfliger and V. Prelog, *Helv. Chim. Acta*, **32**, 544 (1949).

(3) L. F. Audrieth and M. Sveda, *J. Org. Chem.*, **9**, 89 (1944).