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Nature of the Acetylcholinesterase Surface. IV. The Control of Enzymatic Inhibition by Basicity in the Substituted Ethylenediamines[1,2]

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Since previous work has shown that at *p*H 7.4 the diamines $(CH_3)_2NCH_2CH_2N\overset{*}{\frown}(CH_2)_n$ with $n = 4, 5$ (DMPy, DMPi) are powerful inhibitors of the enzyme acetylcholinesterase, the present study was concerned with the relationship between inhibitory strength and the basicity of these amine functions. Determination of the pK_a' values of the nitrogen functions has shown that the basicity of one end of a given inhibitor molecule is markedly affected by protonation of the other end, as observed previously by Gero for closely related diamines. Provisional assignment of these pK_a' values to the individual nitrogen functions in DMPy and DMPi has been made on the basis of enzymatic evidence. A study of the effect of *p*H on percentage inhibition of the enzyme at fixed inhibitor concentration indicates that the monoprotonated species $\rangle\overset{+}{N}—CH_2CH_2—\ddot{N}\langle$ is the effective form of the inhibitor in the *p*H range 5.5–7.5, and that the fraction of this species present on varying the solution *p*H is an important factor in DMPi inhibition.

In previous papers[3a,b] it has been noted that substituted ethylenediamines of the type

$$(CH_3)_2NCH_2CH_2\overset{*}{N}\frown(CH_2)_n$$

I, $n = 4$; II, $n = 5$

(compound I = dimethylaminoethylpyrrolidine, DMPy; compound II = dimethylaminoethylpiperidine, DMPi), where $\overset{*}{N}$ is part of a pyrrolidine or piperidine ring to ensure high activity, are powerful competitive inhibitors of the enzyme acetylcholinesterase. The comparative studies to establish the relative order of inhibitor strength as a function of structure were carried out at constant *p*H (7.4), and consequently gave little information as to the ionic state of the two nitrogen functions in the molecular species responsible for the enzymatic inhibition. Knowledge of the nature of these effective ionic species could lead to a more detailed picture of the enzymatic sites engaged in the competitive binding of these inhibitors and of the natural ionic substrate acetylcholine (AC).

Accordingly, the present work was concerned with: (1) the evaluation of the pK_a' values for both nitrogen functions of compounds I and II to provide *p*H *vs.* ionic state data; (2) the determination of the experimental *p*H *vs.* inhibitory activity curve for the representative compound DMPi over the *p*H range 5.5–7.5; and (3) conclusions in regard to the precise ionic form of the inhibitor effective over this *p*H range, with the consequent bearing on the nature of the adsorptive sites themselves.

Discussion

Determination of pK_a' Values.—Purified samples of the solid dihydrochlorides of I and II were titrated in aqueous solution at 25° with standard base. The pK_a' values were obtained from the titration curves essentially according to the techniques summarized by Hitchcock.[4] It was observed in the course of early titrations that the pK_a' values of these bases are markedly influenced by the ionic strength of the medium,[5] so that it was essential to conduct each amine titration in the presence of enough salt (NaCl used) to minimize variations in μ produced in the course of the run. Determinations of pK_a' values for each amine salt were made at many initial concentrations, over the ionic strength range $\mu = 0.0383$ to 0.1926 for pK_1' and $\mu = 0.0542$ to 0.1853 for pK_2', with the results leading to the following relationships (at 25°) between the acid dissociation constants and $\sqrt{\mu}$.

$$\begin{aligned} &\text{Compound I: } pK_1' = 5.97_7 + 0.937\sqrt{\mu} \pm 0.004 \\ &\text{(DMPy)} \quad pK_2' = 9.43_9 + 0.191\sqrt{\mu} \pm 0.014 \\ &\text{Compound II: } pK_1' = 5.97_5 + 0.839\sqrt{\mu} \pm 0.013 \\ &\text{(DMPi)} \quad pK_2' = 9.40_3 + 0.236\sqrt{\mu} \pm 0.002 \end{aligned} \tag{1}$$

A typical *p*H titration curve for DMPi dihydrochloride is shown in Fig. 1, while the linear relationships between pK_a' values and $\sqrt{\mu}$ for this compound are shown in Fig. 2.

Since the enzymatic inhibition studies were carried out in phosphate media with added NaCl to keep the ionic strength approximately constant at $\mu = 0.143$, the pK_a' values at this value of μ were calculated and are given in Table I below. Also included in Table I are some literature values of pK_a' for certain other amines and diamines of immediate interest.

It is seen from the representative list of compounds in Table I that, neglecting variations introduced by lack of control of ionic strength of the media, *both* the aliphatic tertiary monoamines VI–VIII and the N-substituted heterocyclic monoamines IX–XI display pK_a' values in the range 9–11. In sharp contrast (compounds I–IV) the two types of amine functions coupled in a single substituted ethylenediamine molecule show a sharp alteration in one of the pK_a' values (down to about 6), after the other N function ($pK_a' \sim 9.5$) is already protonated. That this alteration in pK_a' is of the same magnitude for both the heterocyclic and non-cyclic amine moieties of the diamine is shown by comparison of III and IV with I and II, and V with III and IV.

(1) The opinions in this paper are those of the authors and do not necessarily reflect the views of the Navy Department.

(2) Presented in part at the National Meeting of the American Physiological Society, San Francisco, Calif., April 10–15, 1955.

(3) (a) S. L. Friess and W. J. McCarville, THIS JOURNAL, **76**, 1363 (1954); (b) **76**, 2260 (1954).

(4) D. I. Hitchcock in "Chemistry of the Amino Acids and Proteins," C. L. A. Schmidt, ed., Second Ed., C. C. Thomas, Springfield, Ill., 1949, Chapter XI, pp. 604–609.

(5) Notable dependence of the strength of heterocyclic bases on total ionic strength was also noted in the work of W. F. K. Wynne-Jones and G. Salomon, *Trans. Faraday Soc.*, **34**, 1321 (1938).

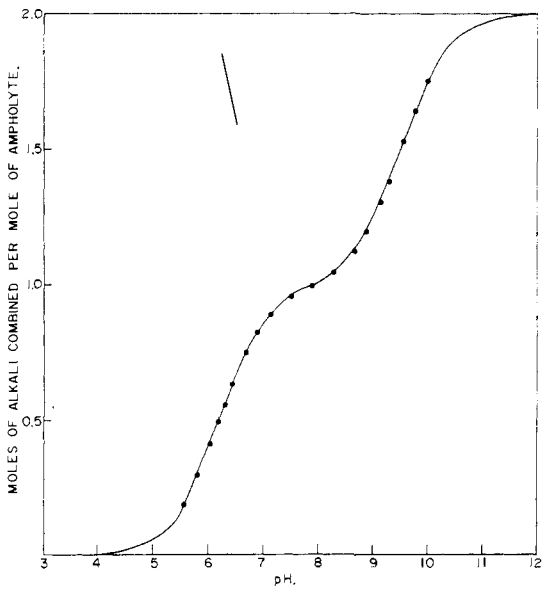

Fig. 1.—Potentiometric titration of DMPi dihydrochloride. The points are experimental, and the solid curve is calculated using the observed pK_a' values.

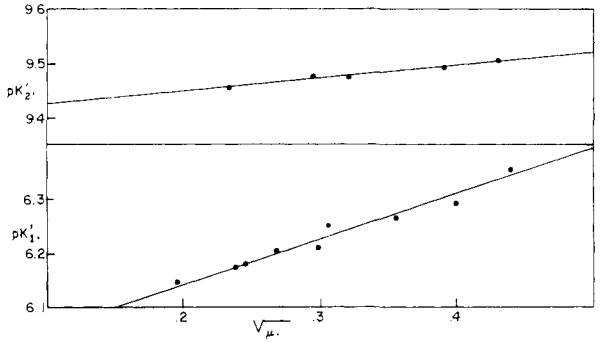

Fig. 2.—DMPi dihydrochloride; pK_1' and pK_2' *vs.* square root of ionic strength.

TABLE I

pK_a' VALUES FOR AMINES AND DIAMINES

Temp., 25°; solvent, water unless otherwise specified

Compound	pK_a'	
I[a]	6.33	9.51
II[a]	6.29	9.49
$C_5H_{10}NCH_2CH_2NC_5H_{10}$ (III)[b]	6.25	9.47
$C_4H_8NCH_2CH_2NC_4H_8$ (IV)[b]	6.30	9.47
$(Et)_2NCH_2CH_2N(Et)_2$ (V)[b]	6.18	9.55
$(CH_3)_3N$ (VI)[c]		9.80
$(C_2H_5)_3N$ (VII)[c]		10.74
$CH_3N(C_2H_5)_2$ (VIII)[c]		10.34
$C_4H_8NCH_3$ (IX)[d]		10.14
$C_5H_{10}NCH_3$ (X)[e]		8.99
$C_5H_{10}NC_2H_5$ (XI)[c]		10.41

[a] Evaluated at $\mu = 0.143$. [b] A. Gero, THIS JOURNAL, **76**, 5158 (1954). [c] N. F. Hall and M. R. Sprinkle, *ibid.*, **54**, 3469 (1932). [d] L. C. Craig and R. M. Hixon, *ibid.*, **53**, 4367 (1931). [e] Obtained in 60% (wt.) methanol–water; W. F. K. Wynne-Jones and G. Salomon, *Trans. Faraday Soc.*, **34**, 1321 (1938).

Consequently, from the magnitudes of the pK_a' values alone for compounds I and II, it is not possible to assign a given pK_a' value to a given functional grouping. However, it seems likely that the $pK_a' \sim 9.5$ value may be assigned to the Me_2N– function in compounds I and II, with the $pK_a' \sim 6.3$ value then being ascribed to the heterocyclic end, on the basis of the following enzymatic evidence. At *p*H 7.4, where the nitrogen function with $pK_a' \sim 9.5$ in compounds I and II would be about 95% protonated and the second function essentially tertiary, both I and II are highly effective acetylcholinesterase inhibitors with enzyme–inhibitor dissociation constants[3a] (K_I) of the order of 10^{-8}. The slightly more powerful quaternary derivatives $Me_3\overset{+}{N}CH_2CH_2\overset{+}{N}(CH_2)_n$ ($2X^-$) also have K_I values of this same order of magnitude, pointing to a similar charge distribution in the quaternized and non-quaternized inhibitor species at *p*H 7.4, with I and II assuming the corresponding positive charge in the form $Me_2\overset{+}{N}HCH_2CH_2{=}\overset{+}{N}(CH_2)_n$ to furnish the charged, polymethylated nitrogen for strong binding at the anionic site. In contrast to these derivatives, the methiodide of compound III in which the *heterocyclic* nitrogen is positively charged has a K_I value[6] of about 1×10^{-6} at *p*H 7.4. This marked decrease in inhibitory power, as reflected in a K_I value greater by 10^2 than those for I and II, is also in accord with the possibility of a non-charged heterocyclic nitrogen in the reactive inhibitory species of I and II at *p*H 7.4. Accordingly, as a reference point in further discussion of the *p*H effect on strength of inhibition, the dimethylamino group in I and II will be taken as the more basic of the two amine functions.

Inhibitor Activity as a Function of *p*H.—DMPi (compound II) displays high inhibitory strength at *p*H 7.4 and 25.12°, with a K_I value[7] of 6.4×10^{-8}. Study of its inhibitory power has now been extended to cover the *p*H range from 7.5 down to 5.5,[8] with the power of a fixed total inhibitor concentration being studied relative to the intrinsic hydrolytic activity of the enzyme at each *p*H level. The results of these inhibition runs on the enzymatically catalyzed AC hydrolysis, in dilute phosphate buffer at a total ionic strength of 0.143, are indicated in Table II.

TABLE II

DMPi INHIBITION OF AC HYDROLYSIS AT VARYING *p*H

25.12 ± 0.03° $(Substrate)_0 = 3.35 \times 10^{-3}$ *M*

$\mu = 0.143$ (DMPi) $= 7.93 \times 10^{-7}$ *M*

(Enzyme) $= 7.5 \times 10^{-6}$ mg. protein/ml.

*p*H	Inhibition of enzyme, %	% of monoprotonated form $\overset{+}{N}$—CH_2CH_2—N
7.42 ± 0.02	53 ± 2	93.1 ± 1.4
7.07	34 ± 4	85.7 ± 1.4
6.53	24 ± 4	63.4 ± 1.1
6.02	15 ± 4	34.8 ± 0.7
5.72	1 ± 2	21.1 ± 0.4

(6) S. L. Friess and H. D. Baldridge, unpublished observation.

(7) Calculated by the method of P. W. Wilson, "Respiratory Enzymes," H. A. Lardy, ed., Burgess Publ. Co., Minneapolis, Minn., 1949, p. 24.

(8) Effectively, a lower limit for any sustained stability of the enzyme; *cf.* M. A. Rothenberg and D. Nachmansohn, *J. Biol. Chem.*, **168**, 223 (1947).

Also included in Table II are the percentages of DMPi present in the monoprotonated form (taken as $Me_2\overset{+}{N}HCH_2CH_2N$⟨⟩) at the various *p*H levels, with the estimated uncertainty in *p*H value being taken at ±0.02 unit in the calculation using the observed pK_a' value of 6.292. Over this *p*H range, the pK_a' value assigned to the dimethylamino function (9.492) indicates that over 95% of this end of the molecule is positively charged and available for interaction with the anionic[9] site, so that changes in inhibitory activity below *p*H 7.4 can be observed as a function of changes in ionic state of one end of the molecule only (taken here as the heterocyclic end). The % inhibition column of Table II is based on the observed rate of catalyzed AC hydrolysis, at the fixed inhibitor and initial substrate concentrations and a given *p*H, relative to the uninhibited reaction rate given by the enzyme–substrate system at that *p*H.

It is seen from Table II that, with one end of the inhibitor completely charged and the other end becoming progressively more protonated as the *p*H is lowered, the effectiveness of the inhibitor suffers a steady drop. The functional relationship between the percentage of inhibitor in the quaternary–tertiary form (⟨⟩$\overset{+}{N}CH_2CH_2\ddot{N}$⟨⟩) and the resulting effect on enzyme inhibition is indicated by the sigmoid-type curve of Fig. 3.

It seems probable that this drop in effectiveness of DMPi with lowering of *p*H can be accounted for by contributions from two effects: (1) the decreasing fraction of the monoprotonated form $\overset{+}{N}CH_2CH_2\ddot{N}$⟨, in which the localized electron pair on nitrogen may be essential[3a] for effective binding to the esteratic site of the enzyme; and (2) an associated decrease in response of the *enzyme* to this locus of high electron density in the inhibitor, which is unaccounted for in the percentage inhibition ratios that already include the decreased catalytic response of enzyme to substrate molecules. In support of the first of these effects is the sigmoid nature of the graph of *p*H *vs.* percentage inhibition by DMPi, also shown in Fig. 3. Here, an index of the resemblance of the *p*H–inhibition curve to the titration curve for the second nitrogen function of the diamine is the presence of the inflection point in the former at *p*H ~ 6.55, which is in fair agreement with the pK_a' value of 6.29 at the inflection point of the latter. This comparison is essentially between the *p*H value to attain roughly half the maximum inhibitory activity under the conditions of Fig. 3, and the *p*H value for half-conversion to the doubly charged form of DMPi. The closeness of these values implies at least some contribution of effect (1) to the drop of potency of DMPi with decreasing *p*H. Also supporting this idea that lowering the *p*H produces a relatively ineffective dipositive inhibitor ion are literature observations (F. Bergmann and others) that diquaternary ions such as hexamethonium^{++} ion are rather poor inhibitors of the enzyme. These observations are in accord with the previous working postulate[3a] that the effectiveness of these diamines is related to the requirement of a locus of relatively high electron density in the inhibitor species for effective binding at the esteratic site of the enzyme.

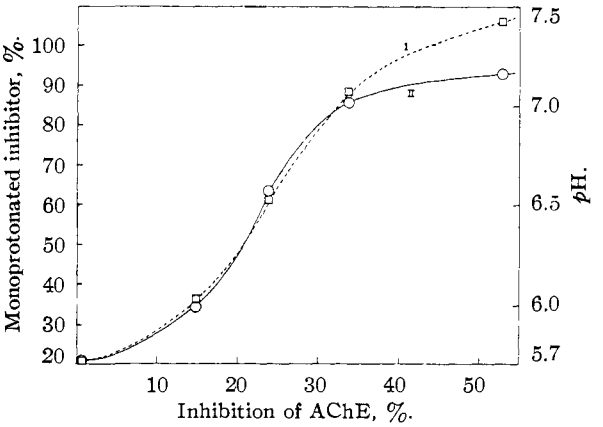

Fig. 3.—Curve I, DMPi inhibition as a function of *p*H; curve II, DMPi inhibition as a function of the fraction of monoprotonated inhibitor present.

However, evidence also can be cited pointing to an intrinsic decrease in response of the enzyme to a given inhibitor as the *p*H is lowered from 7 to 5. For example, Bergmann and Shimoni[10] found that for many monoquaternary inhibitors whose ionic structures are independent of *p*H, the inhibitory effectiveness still fell off with lowering of *p*H. This effect was attributed to neutralization of the anionic site of the enzyme with decreasing *p*H.

Consequently, it would appear that a valid interpretation of all segments of the *p*H–inhibition curve of Fig. 3 for DMPi requires the operation of *p*H effects on both the inhibitor and enzyme molecules.

Experimental

Determination of pK_a' Values.—The dihydrochlorides required for the potentiometric titrations were prepared by passage of anhydrous hydrogen chloride into solutions of the pure amines in absolute ether. The salts were recrystallized twice from absolute methanol and dried at room temperature *in vacuo* for 24 hours. In both cases, the dihydrochlorides sublimed above 200° without melting.

The potentiometric titrations were carried out using a Beckman model G *p*H meter equipped with glass and calomel electrodes. The 5-ml. cell was contained in a water jacket through which water was circulated from a bath maintained at 25.0 ± 0.2°. The solution to be titrated contained about 0.1 millimole of amine dihydrochloride in 3 ml. of distilled water to which sodium chloride was added to adjust the ionic strength of the solution. To this solution were added small aliquots of 0.9316 *N* sodium hydroxide from a microburet, and the *p*H of the resulting solution determined after each addition. Constant stirring was employed.

From these volumetric and *p*H data, the apparent ionization constants of the two substituted ammonium ions were calculated. A review of methods of treating such data is given in reference 4. A curve was plotted relating volume of alkali added and *p*H. The data found to lie on the two straight portions of the titration curve, *i.e.*, the regions of 0.5 and 1.5 moles of alkali added per mole of amine dihydrochloride, were used in calculating the respective apparent ionization constants. These two sets of data were treated

(9) For a description of the postulated duality of sites on this enzyme see: D. Nachmansohn and I. B. Wilson, *Adv. in Enzymol.*, **12**, 259 (1951).

(10) F. Bergmann and A. Shimoni, *Biochim. et Biophys. Acta*, **9**, 473 (1952).

as separate titrations since it was found that the points corresponding to 0.5 and 1.5 equivalents of added base differed by over 3 pH units. The apparent ionization constants were taken as the slopes of the least squares lines in these regions according to the equation

$$(H^+) = K'(HA)/(A^-)$$

where (H^+) is furnished by the pH data and

$$\frac{(HA)}{(A^-)} = \frac{\text{(vol. of alkali for complete neutralization)} - \text{(vol. added)}}{\text{(volume of alkali added)}}$$

Essentially the same results are obtained by averaging the data derived from the following equation for each experimental point.

$$pK' = p\text{H} - \log((A^-)/(HA))$$

The pK' values thus determined were plotted against the square root of the ionic strengths of the corresponding solutions, as shown in Fig. 2.

A curve, Fig. 1, relating pH and the moles of alkali combined per mole of ampholyte was calculated at the ionic strength of a typical DMPi dihydrochloride titration by means of the expression

$$\frac{\text{moles of alk. comb.}}{\text{moles of ampholyte}} = \frac{(A^-)}{(HA + A^-)} = \frac{K_1'(H^+) + 2K_1'K_2'}{(H^+)^2 + K_1'(H^+) + K_1'K_2'}$$

The experimental points are shown plotted along this curve for comparison.

Enzymatic Rate Determinations.—Rate runs in the presence and absence of inhibitors were made as previously described.[3] Duplicate runs were carried out at each pH level for both the inhibited and uninhibited enzymatic hydrolyses, with agreement in rates in each pair of duplicates found to be within 5%. All AC hydrolyses were in the presence of 0.01 M Mg^{++} ion, with the total ionic strength for each phosphate buffer adjusted to 0.143 in final reaction mixtures by means of added sodium chloride. Initial substrate concentrations were fixed at the optimum value of 3.35×10^{-3} M, using twice-recrystallized acetylcholine chloride. The stock enzyme preparation used in these kinetic runs assayed at 2.75×10^5 μmoles AC hydrolyzed/hr./mg. protein/ml. and was obtained by fractionation of the electric organ of *Electrophorus electricus* according to the method of Rothenberg and Nachmansohn.[3] Enzyme solutions aged[11] in at least 1:100 dilution for a week or more were used as secondary stock solutions, from which final aliquots were taken for the actual runs.

DMPi solutions were freshly prepared before use, from redistilled free diamine.[3] Kinetic runs (at 25.12 ± 0.03° in a reaction volume of 3.20 ml.) employing inhibitor gave rates at the various pH levels which were compared to the corresponding hydrolytic activities of the uninhibited enzyme at these same pH values, in calculation of the respective percentage inhibition figures. Estimated errors in these v_i/v inhibition ratios were based on the observed precision of duplicate determinations.

Acknowledgment.—We are indebted to Mr. C. W. Coates and Dr. H. S. Polin for their generous aid in procurement of electric eel tissue and its initial extracts.

(11) For the desensitization of enzyme to Mg^{++} on aging see: S. L. Friess, I. B. Wilson and E. Cabib, THIS JOURNAL, **76**, 5156 (1954).

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Acetylcholinesterase: Enthalpies and Entropies of Activation[1]

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The maximum velocities and Michaelis-Menten constants have been measured as a function of temperature for acetylcholinesterase and a series of acetyl esters of ethanolamine, namely, acetylcholine (I), dimethylaminoethyl acetate (II), methylaminoethyl acetate (III) and aminoethyl acetate (IV). The Michaelis-Menten constants do not change with temperature. Linear Arrhenius plots of the maximum velocities were obtained for the poorer substrates IV and III, but not for II and I which yielded smooth curves approaching very low energies of activation at higher temperatures. These data were interpreted in terms of the previously developed theory of enzyme hydrolysis, specifically in terms of a two-step hydrolytic process involving an acetyl enzyme. Appropriate kinetic relationships were derived and their consequences explored. It is possible to develop a theory of substrate inhibition based in part upon the hydrolysis of acetyl enzyme as a rate controlling step. In general the enthalpy and entropy values in this series are not sufficiently different for substrates II, III and IV to draw conclusions concerning the relationship of these quantities to the specificity characteristics of this enzyme. However, the values for substrate I are very different, in particular the entropy of activation is quite favorable whereas the enthalpy of activation is rather unfavorable compared to the other substrates.

Introduction

Enzyme specificity can be readily analyzed in two categories: (1) specificity in binding the substrate and (2) specificity in the rate with which the enzyme–substrate complex undergoes reaction to yield products and regenerate the enzyme. With a number of enzymes, acetylcholinesterase amongst them,[3] it has been possible to recognize the binding features of a substrate (or competitive inhibitor) and define the corresponding forces which stabilize the enzyme–substrate (or enzyme–inhibitor) complex. It has thus been possible to explain binding. The second category, specificity in the hydrolytic process (in the case of hydrolytic enzymes), deals more directly with the catalytic mechanism. For this reason it is especially desirable to study the hydrolytic specificity for a series of similar substrates and to obtain data at different temperatures so that the enthalpies and entropies of activation may be compared and other mechanistic features possibly revealed. The present study with acetylcholinesterase and a series of acetyl esters of substituted amino alcohols substantiates the conclusion reached in other ways that an acetyl enzyme[4]

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(2) Supported by a Grant from the Rockefeller Foundation. Present address: Instituto de Investigaciones Bioquimicas, Fundacion Campomar, Julian Alvarez 1719, Buenos Aires, Argentina.

(3) I. B. Wilson, "The Mechanism of Enzyme Action," Ed. W. D. McElroy and B. Glass, The Johns Hopkins Press, Baltimore, Md., 1954, p. 642.

(4) I. B. Wilson, F. Bergmann and D. Nachmansohn, *J. Biol. Chem.*, **186**, 781 (1950).