ORGANIC AND BIOLOGICAL CHEMISTRY

[CONTRIBUTION NO. 1298 FROM THE STERLING CHEMISTRY LABORATORY OF YALE UNIVERSITY]

The Effect of Ionic Strength on the Heat of Hydrolysis of Benzoyl-L-argininamide[1]

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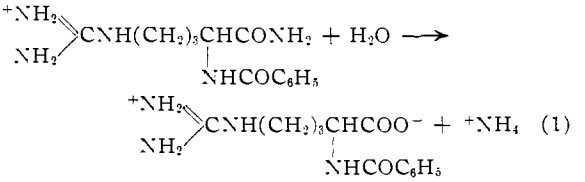
RECEIVED JULY 8, 1955

The heat of the trypsin-catalyzed hydrolysis of benzoyl-L-argininamide to benzoyl-L-arginine and ammonia in dilute aqueous solution has been measured calorimetrically at 25°. A small variation of the heat with the concentration of added salt was observed, ΔH decreasing from -6100 cal. per mole at $\Gamma/2 \approx 0$ to -6700 at $\Gamma/2 = 0.05$ M. Above this concentration no further change was observed, except in the presence of NH_4Cl, which produced a slight rise in apparent ΔH at high concentrations, possibly by affecting the equilibrium of the reaction. Evidence was obtained that the reaction very accurately follows apparent first-order kinetics at substrate concentrations up to 0.06 M, and that the enzyme is weakly inhibited by NH_4Cl.

Introduction

Many reactions in solution which are suitable for direct calorimetric study involve ions. There are energy changes associated with the bringing together or separation of charged particles, and these energy changes depend to some extent on the shielding of the charges by the other ions in solution. Since thermochemical data are determined under a wide variety of conditions, particularly with respect to ionic strength, it is important to investigate the magnitude of the effect of ionic strength on the heats of ionic reactions. Particularly for the complicated ions of interest in biochemical reactions, there is no adequate theoretical basis on which the magnitude of this effect can be predicted. Also, in such cases there are no accurate experimental data extending to low ionic strengths where the effect would be expected to be most pronounced. The present work is primarily an experimental study of this question for a particular reaction, the hydrolysis of benzoyl-L-argininamide (BAA) to give benzoyl-L-arginine (BA) and ammonium ion.

In the pH range where trypsin is active, the hydrolysis of BAA proceeds for the most part in accordance with the stoichiometric equation



$$^{+}NH_2{=}C(NH_2)NH(CH_2)_3CH(NHCOC_6H_5)CONH_2 + H_2O \longrightarrow {}^{+}NH_2{=}C(NH_2)NH(CH_2)_3CH(NHCOC_6H_5)COO^{-} + {}^{+}NH_4 \quad (1)$$

and all the data reported here are corrected to this reaction involving fully charged products. In this reaction a cation produces a zwitterion plus a cation; although one does not ordinarily expect large electrostatic effects in reactions involving zwitterions, in this case the zwitterion has an unusually large dipole moment of approximately 30 Debye units.

Experimental

The calorimetric equipment and method have been described in detail elsewhere.[3] All experiments were performed at 25.00 ± 0.05°.

BAA was prepared from L-arginine[4] by way of benzoyl-arginine ethyl ester, according to the procedure given by Dirr and Späth,[5] and was recrystallized several times and air-dried at room temperature. Kjeldahl nitrogen determinations gave 20.7 ± 0.1% N (calculated for BAA·HCl·H_2O, molecular weight = 331.7, 21.1% N), and gravimetric chloride determinations gave 10.68 ± 0.06% Cl (calculated 10.69% Cl). The material lost 5.70% of its weight on being heated to 105° for 6 hr. (calculated 5.43% H_2O). The ester from which the amide was prepared is quantitatively hydrolyzed by trypsin so that we may assume it to be optically pure; since no racemization is to be expected in the reaction of the ester with ammonia, we may also assume the amide to be optically pure.

Addition of Nessler's reagent indicated that no appreciable hydrolysis of the amide took place in aqueous solution at pH 7 in the absence of the enzyme. It is not possible to determine very small concentrations of ammonia in this way since the reagent forms a precipitate with the amide; however, the very slight coloration observed, which indicated something between 0 and 5% hydrolysis, was independent of the age of the amide solution.

The trypsin was a British Drug Houses preparation, purified by two trichloroacetic acid precipitations, the precipitates being redissolved by dialysis against 0.001 M HCl. All other materials were of reagent grade.

According to Bates and Pinching[6] the pK_a of the ammonium ion at 25° is 9.245 and the standard heat of ionization is 12,500 cal. per mole. We have used these data, even in solutions of high ionic strength, to correct all the observed heat data for the small fraction of the ammonium ion which was in the form of NH_3 at the final pH of each experiment. In the unbuffered experiments there was a small decrease in pH during the experiment. A decrease as large as one pH unit, from pH 7.5 to 6.5, would correspond to the liberation of 3×10^{-7} mole of H^+ per liter, which on reaction with NH_3 would evolve only 4×10^{-3} cal. per liter. Since the actual pH changes were considerably smaller than one unit, we can neglect corrections for the change in pH.

Uncertainty in the Evaluation of ΔH and Reaction Rate.—The interpretation of a given experiment to give values for the heat of reaction and the reaction rate is attended by considerable uncertainty, primarily because the interpretation consists in fitting the experimental data to an equation having three adjustable parameters. If it can be assumed that the reaction is accurately first order, the experimental points should fit an equation of the form

$$r_{obs} - r_0 = \alpha t + (r_\infty - r_0)(1 - e^{-kt}) \qquad (2)$$

where r_{obs} is the integrator output[3] (proportional to electrical energy fed back to the tare calorimeter), r_0 is the extrapolated reading immediately after mixing the reactants at $t = 0$, t is the time since mixing, α is the calorimetric drift rate, $r_\infty - r_0$ is the total change in integrator reading due to the reaction alone (*i.e.*, excluding the effect of calorimetric drift), and k is the rate constant. ΔH is proportional to $r_\infty - r_0$. In actual practice the drift rate is not perfectly con-

(1) This research was aided by grants G179 from the National Science Foundation, and RG3996C from the United States Public Health Service.

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(3) A. Buzzell and J. M. Sturtevant, THIS JOURNAL, **73**, 2454 (1951).

(4) Eastman white label material.

(5) K. Dirr and H. Späth, *Z. physiol. Chem.*, **237**, 121 (1935); M. Bergmann, J. S. Fruton and H. Pollak, *J. Biol. Chem.*, **127**, 643 (1939).

(6) R. G. Bates and G. D. Pinching, *J. Research Natl. Bur. Standards*, **42**, 419 (1949).

stant, so that, except in the case of reactions having half-times of no more than 5 or 10 minutes, it is not satisfactory to determine α after the reaction is complete. Thus, in effect, one has the three independent parameters α, $r_\infty - r_0$, and k (r_0 may be calculated from these three and is thus not independent) in equation 2. We are interested in how wide a range of values of $r_\infty - r_0$ and k may be selected and still have equation 2 agree with the observed readings within the estimated uncertainty of the individual readings. An answer to this question may be obtained in the following somewhat unorthodox way. The change in r (replacing r_{obs} in equation 2) due to changes in the three parameters p_i is

$$\Delta r = \sum \frac{\partial r}{\partial p_i} \Delta p_i \qquad (3)$$

where we do not add the squares as is usual in analyses of errors because we are here interested in manipulations of the parameters rather than chance variations in them. Δr is a function of the time; we select n more or less evenly spaced values of the time, and compute the corresponding values of Δr. Then the requirement that $\Sigma(\Delta r)^2 \leqslant n(\delta r)^2$, where δr is the experimental uncertainty in each value of r, gives an equation which is quadratic in $\Delta\alpha$, $\Delta(r_\infty - r_0)$ and Δk. The range of real values of these quantities which can satisfy the equation is found by considering the equation first as a quadratic in $\Delta\alpha$ and requiring that the discriminant be $\geqslant 0$; this gives an equation not containing $\Delta\alpha$, and in a similar manner from this equation one can obtain an equation in $\Delta(r_\infty - r_0)$ alone. The ranges found for the parameters are independent of the order in which the calculations are carried out. The results obtained by this procedure are summarized for $\Delta(r_\infty - r_0)$ in Table I. The calculation with $n = 10$ leads to $\Delta k/k = \pm 3\delta r/(r_\infty - r_0)$. Since actual observations seldom went below 15% reaction, it may be concluded that $\Delta(r_\infty - r_0) = 10\delta r$ leads to a reasonable estimate of the uncertainty in ΔH. An estimate of δr should include the effect of drift changes, as observed with no reaction proceeding in the calorimeter, as well as of the short time fluctuations in r due to random causes such as noise from the thermometer bridge, and calorimetric imperfections. We estimate that $10^5\delta r = \pm(25 + 5\tau)$ cal., where τ is the duration of the experiment in hours. All uncertainties listed below for individual ΔH values have been calculated in this way. The uncertainties in rate constants due to uncertainties in enzyme concentrations are much larger than those due to δr.

TABLE I

ESTIMATED UNCERTAINTY IN $(r_\infty - r_0)$

n	Range of reacn. included, %	$\pm\Delta(r_\infty - r_0)/\delta r$
3	50–87	26
4	50–94	16
6	30–87	13
8	30–94	13
10	15–94	9.6

Results

Experiments at Low Ionic Strength.—Several experiments were run at low values of the ionic strength $\Gamma/2$, some buffered and some unbuffered. Enzyme concentrations were of the order of 1 mg. of protein per ml. The data for these experiments are summarized in Table II. The ionic strengths listed in the third column include the substrate and all other ionized components except the protein. The initial substrate concentrations are given in the fourth column, and the final values of the pH in the fifth column. The corrected values for the heat of hydrolysis and the computed uncertainties are listed in the sixth and seventh columns.

Experiments in Sodium Chloride Solutions.—Table III gives the data for reactions carried out in NaCl solutions, at ionic strengths up to 2 M. No effect of salt concentration is evident in these data.

TABLE II

THE HEAT OF HYDROLYSIS OF BAA IN SOLUTIONS OF LOW IONIC STRENGTH

$\Gamma/2$, mole per liter	$[S]_0$, moles per liter, $\times 10^4$	Final pH	Added salt or buffer	$-\Delta H$, cal. per mole	Calcd. uncertainty, cal. per mole
0.00057	4.89	6.50	Phosphate	5930	±250
.00057	4.89	6.50	Phosphate	5940	250
.00065	5.97	6.60	None	5820	280
.00065	5.97	6.60	None	6180	220
.00081	7.91	6.82	NH_4Cl	6210	170
.00247	4.65	6.62	Phosphate	6390	370
.00271	6.61	6.78	NH_4Cl	5930	210
.00271	6.61	6.78	NH_4Cl	6250	210
.00282	7.66	7.01	NH_4Cl	6720	170
.00401	4.34	7.13	Phosphate	5710	270
.00401	4.34	7.13	Phosphate	5790	270
.01041	3.86	7.15	NH_4Cl	6390	330
.01060	5.46	6.81	NaCl	6380	240
.01060	5.46	6.82	NaCl	6510	240
.01081	7.91	7.16	NH_4Cl	6750	170
.01256	4.83	6.45	Phosphate	6030	280
.01256	4.83	6.45	Phosphate	6350	260
.0406	5.79	7.15	NH_4Cl	6550	280
.0506	4.77	7.30	THAM[a]	6700	210
.0506	4.77	7.30	THAM	7000	220
.0643	92.9	7.43	THAM	6730	80
				Root mean square	±250

[a] Tris-(hydroxymethyl)-aminomethane.

TABLE III

THE HEAT OF HYDROLYSIS OF BAA IN SOLUTIONS CONTAINING NaCl

$\Gamma/2$, mole per liter	[THAM], moles per liter	Final pH	$[S]_0$, moles per liter, $\times 10^4$	$-\Delta H$, cal. per mole	Calcd. uncertainty, cal. per mole
0.138	0.100	7.41	81.5	6660	±70
.550	.050	7.30	4.71	6460	280
.550	.050	7.30	4.71	7020	280
.550	.050	7.30	4.68	7370	260
.550	.050	7.30	4.68	7430	270
2.001	10^{-4}	6.67	6.27	6250	210
2.001	0	6.55	5.31	6310	230
2.001	0	6.55	5.31	6340	230
2.001	5×10^{-4}	6.40	6.28	6370	270
2.001	0	6.49	6.51	6420	190
2.001	2×10^{-5}	6.67	6.31	6550	210
2.001	0.005	6.96	6.18	6870	250
			Mean	6650	
			Std. dev. from mean	±410	
			Std. error of mean	±120	

Experiments in Ammonium Chloride Solutions.—The effect of NH_4Cl was investigated in a series of runs, the results of which are presented in Table IV. Part of the reason for having used NH_4Cl is the fact that its weak buffering action at pH 7 helps to stabilize the pH without introducing the need for a buffer correction to the heat data. In some of these experiments, as indicated in the table, a small amount of $CaCl_2$ was added to stabilize the enzyme.

Discussion

Variation of ΔH with Ionic Strength.—The data of Table II, plotted in Fig. 1, indicate a dependence

TABLE IV

THE HEAT OF HYDROLYSIS OF BAA IN SOLUTIONS CONTAINING NH_4Cl

$[NH_4Cl]$, mole per liter	$[CaCl_2]$, mole per liter	Final pH	$[S]_0$, moles per liter, $\times 10^4$	$-\Delta H$, cal. per mole	Calculated uncertainty, cal. per mole
0.150	0.005	6.50	4.59	6500	±270
.200	0	7.43	5.73	6470	230
.241	.005	7.24	4.92	6100	270
.400	0	7.40	2.38	6650	540
.400	0	7.38	4.28	6350	290
.400	0	7.40	12.12	6760	130
.400	0	7.43	5.55	6380	290
.640	.005	7.34	4.59	6590	270
.800	0	7.42	1.57	6040	800
.800	0	7.44	17.66	6430	110
.960	0	7.42	3.95	6640	430
.960	0	7.39	5.41	5580	310
1.280	.005	7.33	4.92	5910	270
1.440	0	7.30	7.27	5870	300
1.440	0	7.30	7.27	5860	300
1.600	0	7.31	7.36	5660	190
1.600	0	7.31	7.36	5150	190
1.760	0	7.35	6.73	5880	230
1.760	0	7.35	6.73	5400	230
				Root mean square	±330

of ΔH on $\Gamma/2$ which is statistically significant. Applying the least squares criterion, the values of ΔH can be fitted to either of the equations

$$-\Delta H = 6010 + 3200\sqrt{\Gamma/2} \quad (4)$$

$$-\Delta H = 6120 + 12570\,\Gamma/2 \quad (5)$$

with a standard deviation of 270 cal. per mole. The standard errors of the slopes are, respectively, 820 and 3200.

Kirkwood[7] has developed an expression for the excess chemical potential, due to the presence of other ions, of a spherical ion containing an arbitrary distribution of point charges. Application[8] of his treatment to the BAA and NH_4^+ cations and the BA zwitterion gives an expression for the electrostatic contribution to the free energy of reaction 1, which on differentiation with respect to temperature, assuming the internal dielectric constant of the ions to be independent of temperature, leads to an expression for the electrostatic contribution to the heat of reaction 1. Any reasonable selection of molecular parameters leads to the prediction of a dependence of ΔH on the first power of $\Gamma/2$ at low values of $\Gamma/2$ which, however, is of opposite sign and very much smaller magnitude than indicated by equation 5. The experimental data could be described by an expression having a smaller slope than equation 5, but it is not possible to reverse the sign of the slope. No suitable explanation of the observed dependence of ΔH on $\Gamma/2$ can be advanced at this time.

The data for experiments in NH_4Cl solutions (Table IV) of ionic strengths exceeding 0.10 also show an apparent dependence on ionic strength. The data for runs in NaCl solutions (Table III) in the same range of ionic strengths, on the other hand,

(7) J. G. Kirkwood, *J. Chem. Phys.*, **2**, 351 (1934).

(8) J. M. Sturtevant, to be published.

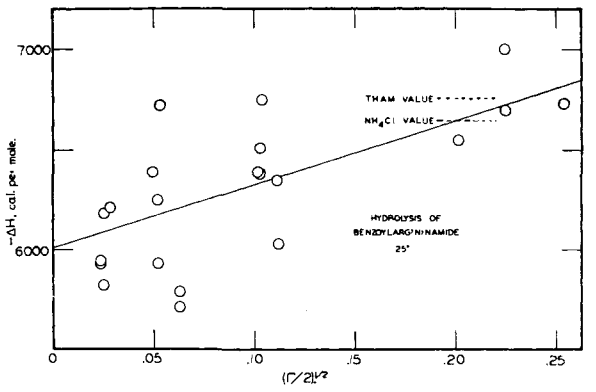

Fig. 1.—The heat of hydrolysis of BAA at low ionic strength. The solid line represents equation 4, and the dashed lines the mean value observed in THAM buffers and the limiting value observed in solutions containing NH_4Cl.

indicate no such dependence of ΔH on $\Gamma/2$ as observed in the NH_4Cl runs. We are therefore led to consider the possibility that the effect of NH_4Cl is actually to change the extent of reaction. If we assume unit activity coefficients, the equilibrium constant for reaction 1 is

$$K' = \frac{[^+BA^-][NH_4^+]}{[^+BAA]} \quad (6)$$

where, as usual, the standard state for H_2O is the pure solvent, and its activity is assumed to be independent of changes in solute concentrations. Then it follows that

$$-\Delta H = -\Delta H_0 + \frac{\Delta H}{K'}[NH_4^+] \quad (7)$$

where ΔH_0 is the heat of reaction at zero NH_4^+ concentration (not the same, of course, as $\Gamma/2 = 0$). The equation

$$\Delta H = -\frac{6760}{1 + 0.117[NH_4^+]} \quad (8)$$

may be derived by the method of least squares, the standard deviation of the ΔH values from this equation being 330 cal. per mole. The value of K' obtained from equation 8 is 8.6 moles per liter, with a standard error of 1.9, which corresponds to $\Delta F^0 = -1270 \pm 300$ cal. per mole. This is an unexpectedly small standard free energy for the hydrolysis of an amide group, and should be checked by independent measurements.[9]

The value of ΔH_0 checks very satisfactorily with the mean value observed in solutions of NaCl (Table III). The average of these two quantities, $\Delta H = -6710 \pm 150$ cal. per mole, is taken as the heat of hydrolysis of BAA at moderate ionic strengths. This value may be compared with $\Delta H = -5840$ cal. per mole for benzoyl-L-tyrosinamide[10] and $\Delta H = -6220$ cal. per mole for glycyl-L-phenylalaninamide.[10]

The Kinetics of the Hydrolysis.—In all the experiments listed in the tables the heat evolution

(9) This probably could be done by experiments using $N^{15}H_4^+$ [J. S. Fruton, R. B. Johnston and M. Fried, *J. Biol. Chem.*, **190**, 39 (1951)], or by calorimetric experiments at 35 or 40°, since the large negative value of ΔH would lead one to expect a considerable decrease in K' with temperature.

(10) J. M. Sturtevant, THIS JOURNAL, **75**, 2016 (1953).

accurately followed apparent first-order kinetics, to 90–95% completion where the effect of small deviations is greatly magnified. The partial loss of enzyme activity during the thermal equilibration period makes it impossible to discuss the kinetic data in terms of Lineweaver–Burk[11] plots, with a few exceptions noted below.

It has been reported by several authors that the hydrolysis of BAA by trypsin follows complicated kinetics. Harmon and Niemann,[12] Schwert and Eisenberg,[13] and Bernhard,[14] all of whom used phosphate buffers at approximately *p*H 7.7, found that the initial rates of hydrolysis depend on initial substrate concentrations in a manner not to be expected for either zero or first-order kinetics, and that the rate in a single experiment does not follow either of the two simple kinetic laws regardless of initial substrate concentration. We are not able to reconcile these observations with the unusually accurate apparent first-order kinetics which we have observed calorimetrically.

Harmon and Niemann[12] and Schwert and Eisenberg[13] concluded that the reaction is subject to product inhibition. A simple formulation of the case of competitive inhibition by product leads to the conclusion[15] that apparent first-order kinetics would be observed only in the special case that the dissociation constant of the enzyme-product complex is equal to the Michaelis-Menten constant for the enzyme-substrate complex. An analogous conclusion is reached on the basis of a more complicated mechanism,[16,17] which includes recognition of the fact that the enzyme catalyzes the reverse reaction in addition to the forward reaction.

Since most of our experiments reported above were either unbuffered, or buffered by NH_4Cl or THAM, whereas previous authors have employed phosphate buffers, we have run some calorimetric experiments in 0.1–0.2 *M* phosphate buffers at substrate concentrations up to 0.06 *M*. The data for such experiments at *p*H 7.5 have in all cases also adhered very accurately to the first-order equation up to more than 95% completion.

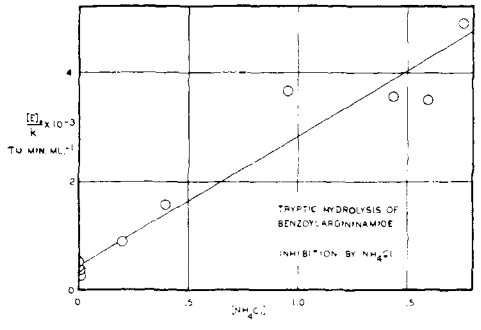

Fig. 2.—Kinetic data showing the inhibitory effect of NH_4Cl in high concentration. The data are plotted in the form suggested by equation 9.

Bernhard[14] concluded that the deviations from zero-order kinetics are to be attributed to self-digestion of the enzyme at a rate which is determined by the initial substrate concentration. That our observation of apparent first-order kinetics cannot be attributed to this factor is shown by the last run listed in Table II. During the entire course of this experiment, which required 8 hr. to reach 95% completion, the activity of the enzyme was determined by the esterase method on samples periodically withdrawn from a duplicate reaction mixture exterior to the calorimeters. Any change in enzyme activity during the reaction amounted to less than 2%. The value of $k/[E]_0$, where $[E]_0$ is the total enzyme concentration expressed in trypsin units[18] (TU) per ml., found in this experiment, with $[S]_0$ = 0.00929 *M*, is 1.9×10^{-3} ml. $(TU)^{-1}$ min.$^{-1}$. The agreement of this figure with the value obtained by extrapolation (see below) of data for runs buffered by NH_4Cl but with $[S]_0$ = 0.0004 to 0.0009 *M* is consistent with true first-order kinetics in the presence of NH_4Cl and THAM, and would indicate a much larger value for the Michaelis–Menten constant than the earlier authors[12-14] reported for systems buffered by phosphate.

The reaction is definitely, but weakly, inhibited by NH_4Cl. In a few runs where rapid thermal equilibration of the calorimeters and solutions was accomplished, it was possible to extrapolate the rate constants observed in the duplicate reactions in the two calorimeters back to the time of makeup of the enzyme solution and thus to remove to some extent the uncertainty in $[E]_0$ resulting from self-digestion of the enzyme. Data for runs of this type performed in solutions of NH_4Cl are plotted in Fig. 2.

In an enzyme reaction which follows first-order kinetics, whatever the cause, and goes nearly to completion, the first-order rate constants in the presence of an inhibitor should follow an equation of the form

$$\frac{[E]_0}{k} = A + B[I] \qquad (9)$$

where [I] is the inhibitor concentration, and *A* and *B* are constants, the interpretation of which depends on the particular mechanism assumed for the reaction. Within the rather large experimental error, resulting mainly from uncertainty in the enzyme concentration, the data follow the requirements of equation 9.

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(11) H. Lineweaver and D. Burk, THIS JOURNAL, **56**, 658 (1934).
(12) K. M. Harmon and C. Niemann, *J. Biol. Chem.*, **178**, 743 (1949).
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(14) S. A. Bernhard, *Biochem. J.*, **59**, 507 (1955).
(15) H. Neurath and G. W. Schwert, *Chem. Revs.*, **46**, 69 (1950).
(16) J. M. Sturtevant, in "The Mechanism of Enzyme Action," edited by W. D. McElroy and B. Glass, Johns Hopkins Press, 1954, pp. 210–214.
(17) R. A. Alberty, THIS JOURNAL, **75**, 1928 (1953).
(18) One trypsin unit hydrolyzes 10^{-6} mole of benzoyl-L-arginine ethyl ester[19] per minute in 0.01 *M* phosphate buffer, 0.1 *M* NaCl, *p*H 8.0 at 25° (ester concentration large enough to insure zero order kinetics).
(19) G. W. Schwert, H. Neurath, S. Kaufman and J. E. Snoke, *J. Biol. Chem.*, **172**, 221 (1948).