[CONTRIBUTION NO. 1471 FROM THE STERLING CHEMISTRY LABORATORY OF YALE UNIVERSITY]

The Heat Content of Bovine Serum Albumin in Acid Solution [1,2]

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When the *p*H of a solution of bovine serum albumin is lowered below about 4, three calorimetrically distinguishable reactions take place. There is first an instantaneous heat evolution which is largely due to the binding of protons by ionized carboxyl groups. This is followed by a very small heat absorption taking place according to first-order kinetics with a half-time of approximately 2 minutes and amounting to 1500 to 3400 cal. per mole of albumin. Finally, if the protein contains a fatty acid impurity, the product of the second reaction slowly loses its acid impurity with the evolution of considerable heat. All three reactions may be reversed by raising the *p*H. It does not appear to be possible to identify the endothermic reaction with any of the other known changes undergone by the protein at low *p*H. The transition from the form with lower apparent heat content to the form with higher heat content takes place within less than one *p*H unit.

It was reported[4] in 1953 that lowering the *p*H of an isoelectric solution of bovine serum albumin (BSA) to about 3.5 induces a reversible reaction which absorbs heat according to first-order kinetics with a half-time of two to three minutes. A detailed calorimetric investigation, to be discussed in the present paper, of the low *p*H behavior of BSA has shown that a total of three calorimetrically distinguishable reactions take place. There is first an essentially instantaneous evolution of heat. This is followed by the endothermic reaction previously reported.[4] Finally, there is observed with some, but not all, samples of the protein a slow reaction which evolves considerable amounts of heat over periods which may exceed 1 hr. These reactions are for convenience denoted as Reaction α, β and γ, respectively.

Experimental Procedures and Materials

Calorimetric Method.—The twin calorimetric apparatus and method have been described[5] previously.

Protein Concentrations.—The concentrations of BSA solutions were determined either by micro-Kjeldahl[6] nitrogen determinations, or by refractive index measurements made with a Phoenix differential refractometer. The nitrogen content of the protein was taken to be 16.07% and its molecular weight to be 67,000. Protein concentrations (after the mixing process in the calorimeters) ranged from 2 to 3.5%.

***p*H Measurements.**—All *p*H measurements were made with a glass electrode and a Beckman Model G *p*H meter, using 0.10 *M* acetic acid, 0.10 *M* sodium acetate buffer[7] of *p*H 4.65 as standard.

Bovine Serum Albumin.—Crystalline BSA was purchased from Armour and Company (lot G-4302) and from Pentex, Inc. (lots A 1201 and B 12016 P). Bovine serum mercaptalbumin was prepared according to the method of Dintzis[8] from Armour fraction V (lot M 12111). Whenever protein solutions were stored for a period of several days, 1 part by weight of merthiolate was added per 10^5 parts of solution. All experiments were carried out with solutions adjusted to $\Gamma/2 = 0.1$ *M* by the addition of NaCl, without any buffer other than HCl, unless otherwise noted. The uncertainty limits given are in all cases ± the standard error of the mean. Errors in the calibration of the calorimeters are negligible in the present experiments.

(1) From the Ph.D. thesis of P. Bro, June, 1956.

(2) This work was aided by grants from the National Science Foundation (G 179) and the United States Public Health Service (RG 3996 C).

(3) General Electric Company Predoctoral Fellow, 1954–1956.

(4) H. Gutfreund and J. M. Sturtevant, THIS JOURNAL, **75**, 5447 (1953).

(5) A. Buzzell and J. M. Sturtevant, *ibid.*, **73**, 2454 (1951).

(6) The authors are indebted to Mr. Robert Miller for the nitrogen determinations.

(7) R. Bates, *Chem. Revs.*, **42**, 1 (1948).

(8) H. M. Dintzis, Ph.D. thesis, Harvard University, 1952.

Results and Discussion

Reaction γ.—Since the last reaction mentioned above proved to be dependent on the presence of trace impurities, it is convenient to consider it first. Figure 1 illustrates reaction γ as observed with mercaptalbumin. Curve A in the figure shows the variation of the apparent heat content of the protein with time measured from the instant of lowering the *p*H from 4.5 to 3.43. A heat evolution amounting to 8 kcal. per mole of protein took place during a period of 2 hr. It was observed that many samples of BSA show the formation of a slight precipitate at low *p*H, and it was thought that this might be connected with the slow heat evolution.

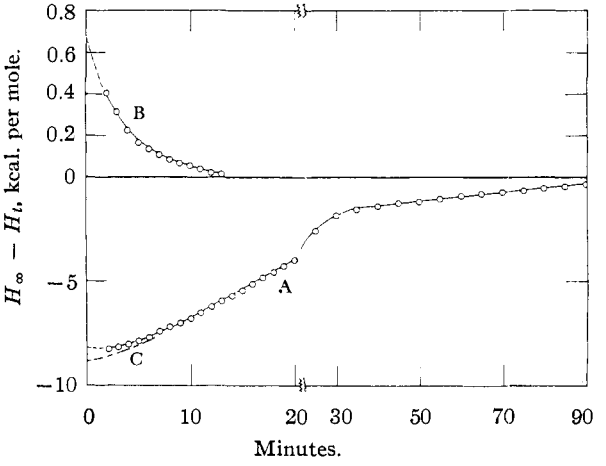

Fig. 1.—The apparent molal heat content of bovine mercaptalbumin in acid solution at 25° as a function of time after lowering the *p*H, relative to the value at infinite time: curve A, heat evolution at *p*H 3.43; curve B, heat absorption at *p*H 3.51 by the same material after pretreatment at *p*H 3.45; curve C, curve A minus curve B.

Accordingly, some of the mercaptalbumin was held at *p*H 3.45 for 5 hr., the solution was centrifuged to remove the insoluble material and the *p*H was readjusted to 4.6. As shown by curve B in Fig. 1, this material now gave no sign of the slow exothermic reaction, showing only the endothermic reaction to be discussed below. Subtraction of curve B from curve A gives curve C. The form of curve C strongly suggests that we are dealing here with a pair of consecutive rather than simultaneous reactions and that the precipitation takes place only after the protein has undergone the endothermic reaction (reaction β).

The precipitate obtained at low *p*H was found to be soluble in ether and in dilute alkali and to be reprecipitated from alkali by acidification. It is less dense than the protein solution, rising to the top during centrifugation. Since stearate is added in the separation of mercaptalbumin from BSA and since it is known that the deionization step in the purification does not completely remove stearate, it is safe to conclude that the precipitate formed during reaction γ is stearic acid.

An experiment, summarized in Fig. 2, was performed which shows that the heat evolution is in all probability due to the complex of processes involved in the liberation of stearic acid from the protein. A sample of BSA showing the normal endothermic reaction of curve A was treated at *p*H 6 with 2.5 moles of stearate per mole of BSA. It was found that the protein now exhibited a heat evolution (curve B) similar to that observed with mercaptalbumin and that this exothermic reaction was consecutive to the endothermic reaction (curve C).

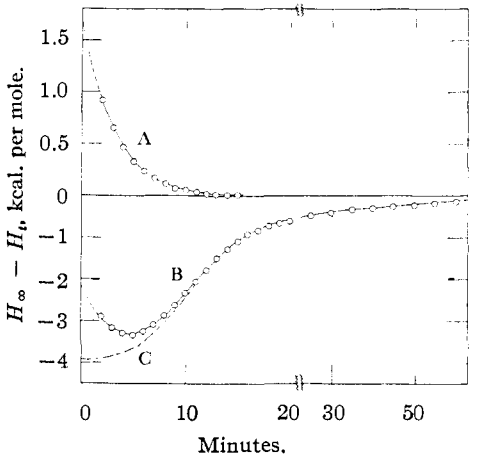

Fig. 2.—Experiment to demonstrate the origin of the slow heat evolution by serum albumin at low *p*H: curve A, normal endothermic reaction at *p*H 3.51; curve B, heat absorption followed by slow heat evolution at *p*H 3.70, shown by the material used for curve A after pretreatment with stearate at *p*H 6; curve C curve B minus curve A.

Evidence was obtained that the heat of reaction γ increases as the *p*H is decreased, presumably because of increase in the amount of stearic acid precipitated. Thus a sample which has been subjected to acid pretreatment at a given *p*H will show reaction γ when taken to a still lower *p*H.

It may be added here that the precipitate which formed on acidification of the samples of Pentex BSA used in these studies was not entirely stearic acid. Some of the precipitate was isolated and found to have an infrared spectrum in chloroform showing definite differences from the spectrum of stearic acid but closely resembling that of a synthetic sample of dinonyl ketone. The decanol used in the crystallization of the protein is prepared under conditions which might lead to the formation of small amounts of dinonyl ketone as an impurity. It has not been ascertained whether the separation of this material from BSA contributes to the observed heat effects.

All results reported below were obtained with protein which either showed no low *p*H precipitation or had been pretreated with acid to remove the precipitate.

Reaction β.—The reaction of greatest interest is reaction β, which is accompanied by absorption of heat. Unfortunately, the situation is very unfavorable for obtaining accurate data for this reaction, since the heat of the reaction is very small, and the heat of mixing accompanying the initiation of the reaction in the calorimeters is 10 to 100 times as large. Furthermore, the rate of the reaction is at the upper limit of the range which the present equipment can handle as non-instantaneous.

The course of a typical experiment at 25° is shown in Fig. 3. The reaction was to be run in calorimeter B, and since it was known that considerable heat evolution would take place during the mixing process, calorimeter A was preheated electrically. At $t = 0$ the valve in calorimeter B was opened to initiate the reaction and further electrical energy had to be supplied to A to bring the calorimeters to the same temperature. Zero temperature difference between the calorimeters was achieved by $t = 1$, and from then on it was necessary to add electrical energy to B to maintain this situation. In this experiment, the total heat of the slow reaction corresponded to a temperature decrease of only 6×10^{-4} deg., and the heat of mixing was twelve times as large. It was not possible to

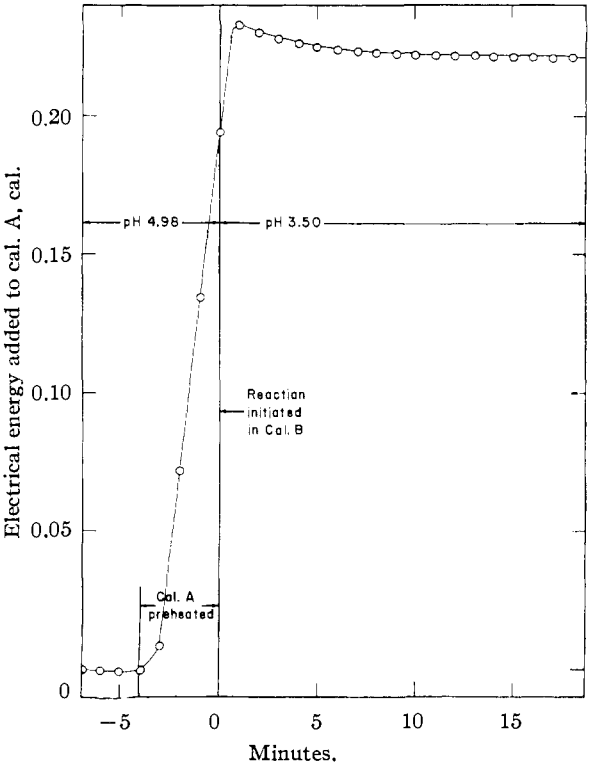

Fig. 3.—The course of a typical calorimetric experiment at 25°. The electrical energy added to calorimeter A (decreasing values indicate electrical energy added to calorimeter B) as a function of time measured from the instant of initiating the reaction in calorimeter B. The calorimeter contained 28 ml. of a 0.341 m*M* solution of BSA.

get useful data pertaining to the slow reaction before $t = 2$, and by then the reaction was nearly half finished.

In spite of these difficulties it is nevertheless possible to obtain data which permit several conclusions to be arrived at concerning the characteristics of the reaction. In the first place, it is reversible. This is indicated by the fact that the reaction is shown by BSA which has previously been exposed to a *p*H considerably lower than that at which the experiment is performed. A more direct proof of reversibility is given in Fig. 4. Two aliquots of

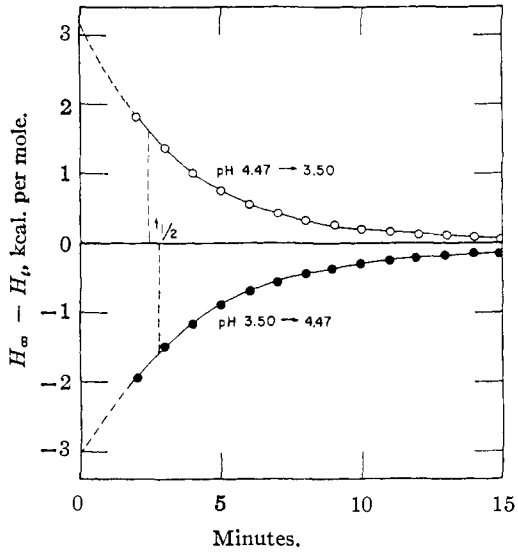

Fig. 4.—Experiments illustrating the reversibility of reaction β. Acetate buffer, $\Gamma/2 = 0.1$, 25°. The apparent molal heat content relative to the final value plotted as a function of time after initiation of the reaction. Open circles, endothermic reaction following lowering of the *p*H from 4.47 to 3.50; filled circles, exothermic reaction following raising of the *p*H from 3.50 to 4.47. The curves are exponentials with rate constants and total heat changes derived from semilogarithmic plots of the heat data.

the same BSA solution were used. One of these was adjusted to *p*H 4.47 and placed in one calorimeter, the other to *p*H 3.50 and placed in the second calorimeter. In the first case the normal heat absorption was observed after the *p*H had been lowered to 3.50 at $t = 0$, while in the second case, a very similar heat *evolution* followed raising the *p*H to 4.47. It is interesting that the rates of the two reactions are nearly the same. These experiments were performed in acetate buffers so that the *p*H in the second one could be raised by adding sodium acetate to avoid the relatively enormous heat evolution which would have accompanied neutralizing HCl with NaOH.

The forward reaction at *p*H 3.50 and the reverse reaction at *p*H 4.47 both followed first-order kinetics with good accuracy. The curves in Fig. 4 are exponentials drawn with rate constants and total heat changes deduced from semi-logarithmic plots of the heat data. In a total of more than 50 experiments, reaction β was found to proceed in good accordance with apparent first-order kinetics.

The heat and rate of reaction β show no significant variation with *p*H over a considerable range, as illustrated in Fig. 5 for data at 15°. Under these conditions, experiments at *p*H 2.76 and 4.33 gave no indication of any endothermic reaction distinguishable from calorimetric lags. It appears

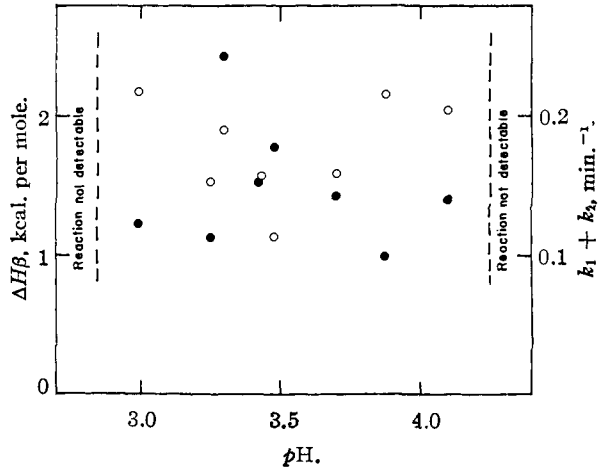

Fig. 5.—Reaction β at 15°: open circles, the sum of the forward and reverse reactions; closed circles, the heat of the endothermic reaction. Neither quantity shows a significant trend with *p*H.

probable that at low *p*H the rate of the reaction becomes too large for observation in the present equipment and that at high *p*H the extent of reaction becomes too small. It has not been possible to define sharply the *p*H range in which the reaction is observable.

Bro, Singer and Sturtevant[9] have described an aggregation reaction which takes place in BSA solutions at low *p*H. It was found that the heat and the rate of reaction β are independent of the presence or absence of low *p*H aggregates. Four runs at 25° with Pentex BSA containing 50% of dimer as deduced from sedimentation studies gave $\Delta H_\beta = 1750 \pm 250$ cal. per mole and $k = 0.30 \pm 0.03$ min.$^{-1}$. Four runs performed on this material after the content of dimer had been reduced to approximately 5% by passage over an ion-exchange column including a thioglycolate section[9] gave $\Delta H_\beta = 1800 \pm 300$ cal. per mole and $k = 0.29 \pm 0.02$ min.$^{-1}$.

The data on the endothermic reaction are summarized in Table I. Comparison of the first two sets of data indicates, as mentioned above, that acid pretreatment of the protein has no effect on the reaction. Comparison of the last set, at 15°, with the first two sets indicates that the heat of the reaction is independent of temperature, which means that there is no significant change in heat capacity during the reaction and that the rate of the reaction is only weakly dependent on temperature. The heat of activation calculated from the rates at 15 and 25° is 8700 cal. per mole.

The heat of reaction observed with samples of Armour BSA is approximately twice as large as observed with Pentex BSA, although the rate of the reaction is the same for both materials. It appears

(9) P. Bro, S. J. Singer and J. M. Sturtevant, THIS JOURNAL, 80, 389 (1958).

TABLE I

RATE AND HEAT OF THE LOW-*p*H ENDOTHERMIC REACTION OF BOVINE SERUM ALBUMIN

Material	Temp., °C.	No. of determinations	*p*H	Ionic strength	$k_1 + k_2$, min.$^{-1}$	$\Delta H\beta$, cal./mole
Pentex, acid pretreated	25	16	2.85-3.94	0.04-0.12	0.31 ± 0.01[a]	1600 ± 110[a]
Pentex, no pretreatment	25	10	3.50-3.89	0.10	0.31 ± .02	1780 ± 100
Armour, initial *p*H 4.5	25	8	3.22-3.50	.10	.29 ± .01	3390 ± 180
	25	2	3.85	.10	.30 ± .04	1750 ± 60
Armour, initial *p*H 3.5	25	3	4.32-4.71	.10	.24 ± .01	−3750 ± 510
Pentex	15	8	3.00-4.10	.10	.18 ± .01	1500 ± 160

[a] Uncertainty limits are ± standard error of mean. No allowance for uncertainties in calorimetric calibrations is necessary.

that, at least with respect to this property, considerable differences between different samples of BSA can be found.

If the reaction is indeed reversible and first order in both directions, the observed rate constant is equal to $k_1 + k_2$, the sum of the forward and reverse rate constants, and the equilibrium constant is the ratio k_1/k_2. The heat data for Armour BSA indicate that the protein is entirely in the low *p*H form at all *p*H values below 3.5 and entirely in the high *p*H form above 4.3. On the other hand, $k_1 + k_2$ appears to be essentially constant over the entire *p*H range. The two runs at *p*H 3.85 indicate that the equilibrium constant is approximately unity at this *p*H. These observations are consistent with the hypothesis that there is a transition in the protein in the neighborhood of *p*H 3.85, as schematically represented in Fig. 6. It is necessary to assume that the transition takes place over a relatively narrow range of *p*H, perhaps by some sort of coöperative mechanism. The dashed portion of the k_1 curve merely suggests one explanation for the non-observability of the reaction at very low *p*H.

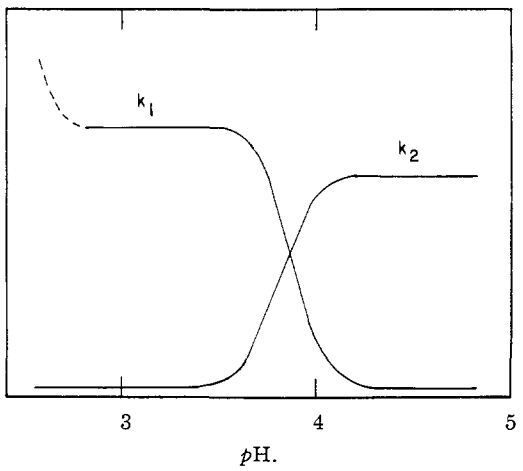

Fig. 6.—Schematic representation of the variation with *p*H of the forward (k_1) and reverse (k_2) rate constants which is consistent with the experimental observations. The dashed portion of the curve for k_1 illustrates a possible explanation for the non-observability of the reaction at very low *p*H.

On the basis of titration and viscosity data, Tanford, Buzzell, Rands and Swanson[10] have concluded that BSA exists in a compact form at *p*H values between 4.3 and 10.5. Just below *p*H 4.3 it changes to an expandable form which then undergoes a continuous expansion which increases with increasing charge and decreases with increasing ionic strength. It is tempting to conclude that the endothermic reaction is identical with this change to an expandable form. This conclusion appears, however, to be untenable since Tanford and Lovrien[11] have found that the uptake of hydrogen ions by BSA at all *p*H values is complete within a few seconds. Furthermore, since the expanded form binds more protons at a given *p*H than the compact form, it would be expected that the small endothermic heat would be overshadowed by an accompanying heat evolution, which should depend on both *p*H and temperature, due to the binding of protons.

As seen earlier, the heat of reaction β at 15° (with Pentex BSA) is essentially constant up to values of the *p*H at least as high as 4.1. In one experiment at *p*H 4.33, no heat absorption was observed. With this same material, in one experiment at *p*H 4.18 at 25° only a very small heat absorption was observed. This apparently small effect of temperature on the *p*H of the transition is consistent with the small apparent heat of ionization deduced by Tanford, Swanson and Shore[12] from titration data, if the reasonable assumption is made that the transition takes place at a constant value of the charge on the protein.

Aoki and Foster[13] have reported an electrophoretic heterogeneity in BSA which they have interpreted in terms of a *p*H-dependent equilibrium between two forms of the protein. From the effects of temperature and *p*H on the equilibrium, they estimated the heat of the reaction to be 3.0 ± 1.5 kcal. per mole and suggested that the reaction leading to a change in electrophoretic mobility is identical with the endothermic reaction. However, it appears unlikely that electrophoretic separation of species which re-equilibrate within a few minutes would be possible.

There is no doubt that important changes in the BSA molecule take place as the *p*H is lowered below approximately 4. However, it is not possible at present to achieve a satisfactory correlation between the various changes in properties reported as taking place in this region of *p*H.

Reaction α.—We are concerned here with the essentially instantaneous processes which follow the addition of acid to a solution of BSA. The

(10) C. Tanford, J. G. Buzzell, D. G. Rands and S. A. Swanson, THIS JOURNAL, **77**, 6421 (1955).

(11) C. Tanford and R. Lovrien, private communication.

(12) C. Tanford, S. A. Swanson and W. S. Shore, THIS JOURNAL, **77**, 6414 (1955).

(13) K. Aoki and J. F. Foster, *ibid.*, **79**, 3385 (1957).

observed instantaneous heats include very small contributions from the heats of dilution of BSA and HCl, and suitable corrections have been applied to the data discussed below. Figure 7 presents a summary of the heats of mixing, expressed as apparent molal heat contents of the protein relative to its state at *p*H 5.0. Heats observed at 25° at closely equal values of the *p*H have been averaged to simplify the plot. In experiments in which reaction β was observed, extrapolation[5] to $t = 0$ was employed to separate the instantaneous heat of mixing from the slow heat absorption. In other cases the heat of mixing includes the heat of any rapid endothermic reaction which took place. The values for the heat of mixing show, for some unknown reason, much more scatter than would be expected on the basis of a great deal of previous experience with these calorimeters.

At 25°, the apparent heat content of BSA immediately after being acidified is essentially independent of *p*H in the range 2.5 to 4.0 and approximately 20 kcal. per mole smaller than at *p*H 5. In contrast, at 15° the heat content decreases steadily down to the lowest *p*H investigated. In going from *p*H 5 to 3, 20 kcal. per mole are evolved at 25° and 100 kcal. per mole at 15°. This indicates that the apparent heat capacity of BSA in solution increases by approximately 8000 cal. mole^{-1} deg.$^{-1}$ between *p*H 5 and 3.

When HCl is added to BSA, hydrogen ions are bound by the protein, together with a much smaller number of chloride ions. It has been estimated that the heat of binding chloride ions by human serum albumin[14] is 0.43 ± 0.54 kcal. per mole. No data are available for the heat of binding chloride ions to bovine serum albumin, but it has been reported[15] that these two proteins are quite similar with respect to chloride binding. In the absence of more definite data, heat effects due to chloride binding will be neglected.

The dashed curve in Fig. 7 gives the increase in protons bound at 25° as the *p*H is lowered below 5.0, as determined by Tanford, Swanson and Shore.[12] Comparison of this curve with the heat content curve at 25° indicates that the carboxyl groups which are protonated between *p*H 5 and 4 have heats of ionization of approximately 0.75 kcal. per mole, while those which are protonated between *p*H 4 and 2.5 have heats of ionization which are vanishingly small. These conclusions are based on the assumption that no instantaneous process other than the binding of protons by carboxylate ions occurs in the *p*H range under consideration.

(14) G. Scatchard, I. H. Scheinberg and S. H. Armstrong, THIS JOURNAL, **72**, 535 (1950).

(15) G. Scatchard, J. S. Coleman and A. L. Shen, *ibid.*, **79**, 12 (1957).

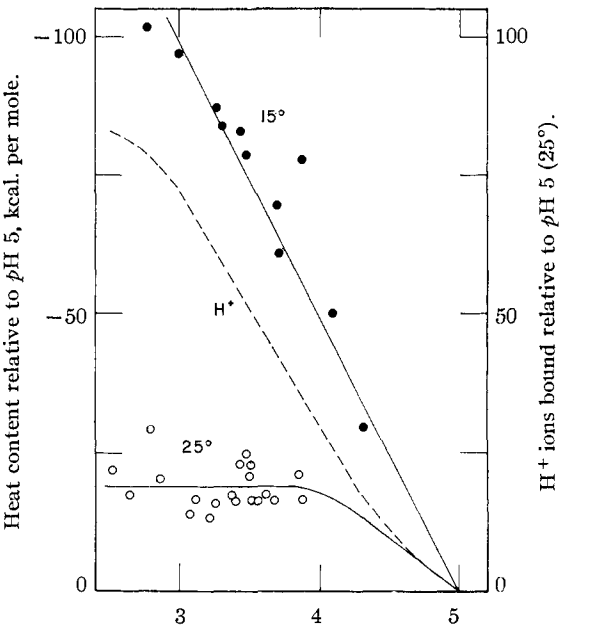

Fig. 7.—The instantaneous heat evolution (reaction α) when solutions of BSA are acidified, expressed as the apparent molal heat content relative to the value at *p*H 5. The dashed curve represents the binding of H^+ ions as determined by Tanford, Swanson and Shore, ref. 12.

The same proton binding curve applies quite well also at 15°, since the apparent heat of ionization[12] is very small. It thus appears that the heats of ionization of the carboxyl groups at 15° decrease from 1.7 kcal. per mole at *p*H 4 to 1.4 at *p*H 3. From titration curves at 5 and 25°, Tanford, Swanson and Shore[12] concluded that the heat of ionization at 15° is about 2000 cal. per mole at *p*H 4.5 and close to zero at *p*H 3. It is probable that the differences between the calorimetric and titrimetric heats of ionization are within the accuracy of the titration data.

The large change in apparent heat capacity mentioned above may be expressed in terms of the ionization reaction. There is a decrease in heat capacity per mole of hydrogen ions dissociated which is practically constant from *p*H 4 to 3 and amounts to 103 cal. per deg. This figure is of the same sign but three times as large as the ΔC_p observed for the ionization of simple carboxylic acids. The decrease in heat capacity as charges are produced is due in large part to electrostriction, and there is no obvious reason to expect the electrostrictive effect to be larger for charges on a protein than for charges on simple molecules. It does not seem possible at the present time to give a satisfactory explanation for these unusually large changes in heat capacity.

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