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Characterization of the Sulfhydryl Groups and the Kinetics of the Heat Denaturation of Crystalline β-Lactoglobulin[1]

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The sulfhydryl groups of native β-lactoglobulin were found to be very unreactive to most sulfhydryl group reagents with the exception of iodine under acid conditions, but upon denaturation by guanidine or heat treatment they are raised to varying degrees of reactivity. Upon heat treatment, a maximum of about 75% of the sulfhydryl groups became available for oxidation by molecular oxygen. Most of the remaining sulfhydryl groups are made reactive to various reagents such as *o*-iodosobenzoate and iodoacetamide but not to molecular oxygen. The number of very reactive sulfhydryl groups (oxidizable by oxygen) which are formed is a function of the time and temperature of the heat treatment and it has been possible to follow the kinetics of the protein denaturation by this method, using the loss in *o*-iodosobenzoate–iodine titer as an indication of the degree of activation. The denaturation reaction follows first order kinetics, and has an energy of activation of about 80,000 calories per mole over the temperature range of 64 to 75°.

Extensive studies[2-5] have clarified the status of the sulfhydryl groups of several proteins, particularly those of egg albumin,[5a] and have demonstrated their increase in reactivity upon denaturation. The state of the sulfhydryl groups of β-lactoglobulin, however, has not been clearly elucidated. Titration with porphyrindin indicates sulfhydryls equivalent to 0.55% cysteine in a guanidine denatured sample.[6] Larson and Jenness,[7] titrating native β-lactoglobulin with a modification of the *o*-iodosobenzoate titration,[8] found -SH groups equivalent to 1.30% cysteine. The cysteine content of the hydrolyzed protein has been reported as 1.11%.[9]

Study of the sulfhydryls of β-lactoglobulin is of practical importance since it is the principal sulfhydryl-containing protein of milk[10]; the activation of sulfhydryl groups by heat has long been considered responsible for cooked flavor characteristics and improved antioxygenic properties of heated milk. The heat activated sulfhydryls of β-lactoglobulin are of further interest in that they are susceptible to oxidation by atmospheric oxygen.[10]

The purpose of this paper is to characterize more thoroughly the reactivity of the sulfhydryl groups of β-lactoglobulin and to demonstrate the effect of heat denaturation upon them. For comparative purposes, some determinations were also made on crystalline egg albumin.

Methods

Amperometric *o*-iodosobenzoate–iodine titrations for protein sulfhydryl groups were conducted by the method of Larson and Jenness.[7] Guanidine hydrochloride was purified as suggested by Greenstein and Jenrette[3]; iodoacetamide was prepared by the method of Anson[11]; nitrogen analyses were performed by a micro-Kjeldahl procedure[10]; and nitroprusside tests were conducted according to the modification of Anson.[12] The β-lactoglobulin was prepared essentially as described by Bull and Currie[13] and egg albumin by the method of Kekwick and Cannan.[14] Both proteins were recrystallized four times. Solutions of the proteins were prepared in phosphate buffer (*p*H either 6.6 or 6.9, $\mu = 0.1$).

Results and Discussion

Nitroprusside Test.—Native β-lactoglobulin in neutral buffer, like native egg albumin, gives a negative nitroprusside test. Both proteins, however, yield positive tests when denatured by 8 *M* guanidine or by heat. Thus both contain potentially reactive sulfhydryl groups.

Reaction with Neutral *o*-Iodosobenzoate.—In the titration method employed,[7] *o*-iodosobenzoate is added to the protein solution at a *p*H near neutrality and the excess *o*-iodosobenzoate is determined by titrating the iodine liberated upon addition of acid and iodide. Either the *o*-iodosobenzoate at neutrality or the iodine in acid solution can serve as oxidant. The sulfhydryl groups of simple compounds such as cysteine or glutathione and those of guanidine denatured egg albumin are oxidized directly by the neutral *o*-iodosobenzoate.[8] However, previous experiments in this Laboratory[7] have shown that in the titration of *native* proteins by this method few, if any, of the sulfhydryls of β-lactoglobulin and only about 10% of those of egg albumin are oxidized by the *o*-iodosobenzoate at neutrality. This conclusion is based on the finding that addition of excess *o*-iodosobenzoate at *p*H 6.6, followed by dialysis to remove the excess, decreased the sulfhydryl content as determined by the regular titration method by 1.5 and 10% for β-lactoglobulin and egg albumin, respectively. It was concluded that in the titration of these native proteins most of the oxidation is accomplished by the iodine liberated in the iodimetric determinations of the excess *o*-iodosobenzoate.

In the present investigation a similar experiment was conducted on heat denatured β-lactoglobulin and heat denatured egg albumin. The protein sols (*p*H 6.6, $\mu = 0.1$) were treated with excess *o*-iodosobenzoate, exhaustively dialyzed against buffer at the same *p*H to remove the excess reagent and finally titrated by the regular procedure. The results, shown in Table I, indicate that heat treatment considerably activates the sulfhydryl groups making them oxidizable by *o*-iodosobenzoate at *p*H 6.6. With β-lactoglobulin the total sulfhydryl content is greatly decreased after heat treatment because of oxidation by atmospheric oxygen during and following heating.[10] Almost all of the remaining groups are oxidizable by *o*-iodosobenzoate at *p*H 6.6. The sulfhydryls of egg albumin are not oxidized extensively by oxygen during and following heat treatment but they are rendered partially oxidizable by *o*-iodosobenzoate at *p*H 6.6.

Reaction with Iodoacetamide and Copper.—Inasmuch as the *o*-iodosobenzoate–iodine titration appears to determine

(1) Paper No. 2778, Scientific Journal Series, Minnesota Agricultural Experiment Station. Data taken from Ph.D. thesis of B. L. Larson, 1951.

(2) M. L. Anson, *Advances in Protein Chem.*, **2**, 361 (1945).

(3) J. P. Greenstein and W. V. Jenrette, *J. Biol. Chem.*, **142**, 175 (1942).

(4) A. E. Mirsky, *J. Gen. Physiol.*, **24**, 709 (1941).

(5) H. S. Olcott and H. Fraenkel-Conrat, *Chem. Revs.*, **41**, 151 (1947).

(5a) L. R. MacDonnell, R. B. Silva and R. E. Feeney, *Arch. Biochem. and Biophys.*, **32**, 288 (1951).

(6) M. L. Groves, N. J. Hipp and T. L. McMeekin, THIS JOURNAL, **73**, 2790 (1951).

(7) B. L. Larson and R. Jenness, *J. Dairy Sci.*, **33**, 890 (1950).

(8) L. Hellerman, F. P. Chinard and V. R. Dietz, *J. Biol. Chem.*, **147**, 443 (1943).

(9) E. Brand, L. J. Saidel, W. H. Goldwater, B. Kassell and F. J. Ryan, THIS JOURNAL, **67**, 1524 (1945).

(10) B. L. Larson and R. Jenness, *J. Dairy Sci.*, **33**, 896 (1950).

(11) M. L. Anson, *J. Gen. Physiol.*, **23**, 247 (1939).

(12) M. L. Anson, *ibid.*, **24**, 399 (1941).

(13) H. B. Bull and B. T. Currie, THIS JOURNAL, **68**, 742 (1946).

(14) R. A. Kekwick and R. K. Cannan, *Biochem. J.*, **30**, 227 (1936).

TABLE I

EFFECT OF PRETREATMENT WITH *o*-IODOSOBENZOATE ON THE REDUCING POWER OF β-LACTOGLOBULIN AND EGG ALBUMIN

Sample[a]	Reducing capacity as per cent. cysteine[b] Untreated	Treated[c]
β-Lactoglobulin		
Native	1.28	1.26
Heated[d]	0.27	0.03
Egg albumin		
Native	1.10	.98
Heated[e]	1.04	.25

[a] All in phosphate buffer (*p*H 6.6, μ = 0.1). [b] Determined by *o*-iodosobenzoate–iodine titration. [c] 0.40 meq. of *o*-iodosobenzoate added per g. of protein and the excess removed by exhaustive dialysis against buffer. The reagent was added to the heated samples immediately after heating. [d] Heated 30 min. at 78°. [e] Heated 15 min. at 75°.

the total sulfhydryl content of both native and denatured proteins it affords a valuable means of determining whether iodoacetamide, copper or other reagents will react with the sulfhydryl groups of a given protein system. To 50-ml. portions of sols of native β-lactoglobulin (1%) or of native egg albumin (0.35%), both in phosphate buffer (*p*H 6.9, μ = 0.1), were added 1.38 g. of iodoacetamide or 1 p.p.m. of copper as copper sulfate (2.5 ml. of a solution containing 0.02 mg. of copper per ml.). Titrations were made by the regular iodosobenzoate procedure on aliquots of the sols at suitable intervals after adding the reagents. Titrations were also made on control sols containing no added iodoacetamide or copper. The results (Fig. 1) indicate that iodoacetamide reacts with a majority of the sulfhydryl groups of native egg albumin at *p*H 6.9. Anson[15] has shown that iodoacetamide reacts with about half of the sulfhydryl groups of this protein at *p*H 9. Iodoacetamide does not react with the sulfhydryl groups of native β-lactoglobulin under these conditions and added cupric ions (1 p.p.m.) do not affect the sulfhydryl titration of either protein.

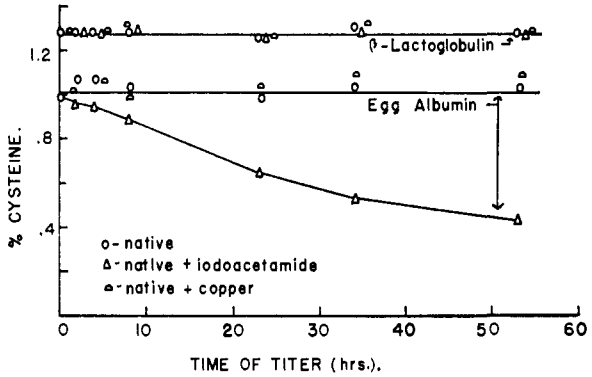

Fig. 1.—Influence of iodoacetamide and copper on reducing power of native egg albumin and β-lactoglobulin. Copper and iodoacetamide were added to separate solutions of the native proteins at *p*H 6.9 and titrations were made by the *o*-iodosobenzoate–iodine procedure at the indicated times after the additions.

The effect of heat treatment on the reactivity of the sulfhydryl groups of β-lactoglobulin to copper and iodoacetamide was next investigated. Figure 2 shows the characteristic decrease in *o*-iodosobenzoate–iodine titer that occurs during and following heat treatment of β-lactoglobulin in the presence of air. Addition of iodoacetamide to the freshly cooled solution rapidly reduced the titer to a lower value indicating that certain sulfhydryl groups not attacked by molecular oxygen do react with iodoacetamide. Copper (1 p.p.m. added before heating) also reduced the titer markedly below the values obtained in its absence.

Reaction with Oxygen.—The decrease in sulfhydryl titer, which occurs upon heat treatment of β-lactoglobulin in air,

(15) M. L. Anson, *J. Gen. Physiol.*, **23**, 321 (1940).

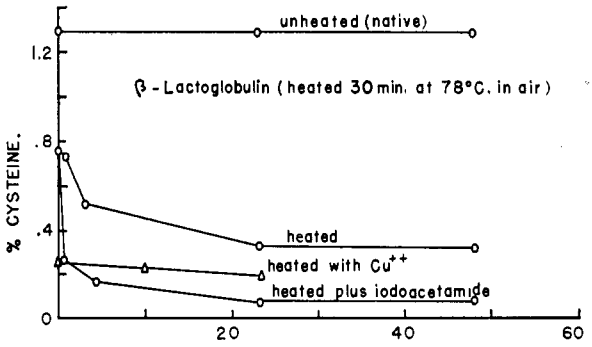

Fig. 2.—Effect of adding iodoacetamide after heating and copper before heating on the decrease in the reducing power of β-lactoglobulin. Samples were titrated by the *o*-iodosobenzoate–iodine procedure at the indicated times after the heat treatment.

apparently involves oxidation by atmospheric oxygen since it is largely prevented if the heat treatment is conducted in an atmosphere of nitrogen.[16] The results in Table II show that the titer not only decreases during heating but that it continues to decrease if the heated sample is held at 5° for periods up to 48 hours. The same final level is reached regardless of whether the heating is conducted in the presence of air; the differences in rate of oxidation doubtless reflect differences in rate of diffusion of air into the sample. In the experiment some of the samples were titrated in the presence of guanidine (8 *M*) as well as in its absence. The data show that guanidine denaturation has little effect on the *o*-iodosobenzoate–iodine titer of either native or heat denatured β-lactoglobulin. This finding lends support to the view that this titration procedure determines all of the sulfhydryl groups of the protein even in the absence of denaturing agents.

TABLE II

THE SULFHYDRYL TITRATION OF β-LACTOGLOBULIN AS AFFECTED BY (A) HEAT TREATMENT IN AIR AND NITROGEN, (B) HOLDING AFTER HEAT TREATMENT AND (C) THE PRESENCE OF GUANIDINE

Time after heating,[a] hr.	Reducing capacity as % cysteine: Heated in air[b] No guanidine	Heated in air[b] 8 *M* guanidine	Heated in nitrogen[b] No guanidine	Heated in nitrogen[b] 8 *M* guanidine
Control—no heating	1.32	1.28	..	..
0 (immediate)	0.83	0.83	1.10	1.13
0.5	.72	..	0.83	..
1	.68	..	.81	..
2	.67	..	..	..
3	.67	..	..	..
24	.39	.42	.40	0.37
48	.31	..	.33	..

[a] Held at 4–5°. [b] Heat treatment at 78° for 30 min. All 1% protein sols in phosphate buffer (*p*H 6.9, μ = 0.1).

If the loss in titer of β-lactoglobulin upon heat treatment is due to oxidation of the sulfhydryl groups by molecular oxygen, it should be possible to demonstrate an uptake of oxygen by the protein during the heating process. An experiment to test this hypothesis was made with the Warburg respirometer. Four-ml. aliquots of 1% β-lactoglobulin sols in phosphate buffer at *p*H 6.9 and μ = 0.1 and containing 0.0, 1.0, and 10.0 p.p.m. of copper as copper sulfate were heated in the apparatus at 69.8° with shaking. Manometer readings were made at intervals to determine the oxygen uptake. The results (Fig. 3) indicate that oxygen is consumed by the β-lactoglobulin during the heating process, and that the consumption is greater if copper is present. After the heat treatment of 240 minutes, the samples were carefully rinsed out of the Warburg flasks and titrated by the regular *o*-iodosobenzoate–iodine procedure. It was then possible to calculate the amount of oxygen taken up as compared to the actual loss in reducing titer. The oxidation of

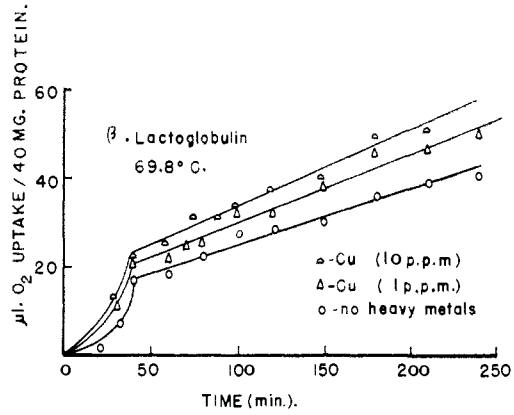

Fig. 3.—Oxygen uptake by β-lactoglobulin during heat treatment. Four ml. of 1% protein sols containing 0.0, 1.0 and 10.0 p.p.m. of copper as copper sulfate was heated in a Warburg apparatus and the total amount of gas taken up was read from the manometers at the indicated times.

a mole of -SH required 0.79 mole of O_2 in the absence of copper and 0.62 mole of O_2 when copper was present. Simple oxidation of a mole of sulfhydryl to disulfide groups would require 0.25 mole of oxygen. Thus it must be assumed that the sulfhydryl groups probably are oxidized beyond the disulfide state in this process. These preliminary results demonstrate the important fact that oxygen is taken up by β-lactoglobulin, an effect which was predicted from the loss in sulfhydryl titer and the influence of oxygen thereon.

Kinetics of the Activation of Sulfhydryl Groups by Heat.—Since heat treatment of β-lactoglobulin results in a loss in sulfhydryl titer as measured by the *o*-iodosobenzoate–iodine method, and since the extent of this decrease depends on the temperature of heating,[10] the concentration of heavy metals, and the availability of oxygen, it should be possible by controlling these factors to determine the kinetics of the heat activation of the sulfhydryl groups of this protein. For this purpose two experiments were made. In both all glassware was cleaned before use with concentrated nitric acid and water redistilled from glass in order to reduce heavy metal contamination.

In the first experiment, about 50 ml. of 1% β-lactoglobulin sol in phosphate buffer (*p*H 6.9, $\mu = 0.1$) was heated in a flask and at suitable intervals (10, 20, 30 and 50 minutes) two 5-ml. aliquots were withdrawn, one of which was titrated immediately and the other held in a 50-ml. erlenmeyer flask at 4–5° for 48 hours to allow oxidation of activated groups to take place before titration. The data from this experiment (Fig. 4) illustrate the decrease in titer over a 48-hour period. The higher the temperature of heating, the lower is the immediate titer and the greater is the decrease on standing. The immediate titration values show little consistency but the 48-hour titration values are in line with the extent of the heat treatment. This is undoubtedly due to the fact that the oxygen availability plays an important part in the immediate titration values, but after 48 hours, if ample opportunity is given for oxidation of the activated groups, the titer reflects the amount of heat treatment the sample has received.

In the second experiment, sols of the β-lactoglobulin were prepared at a concentration of 2% in phosphate buffer (*p*H 6.9, $\mu = 0.1$). Tubes containing 3 ml. of the buffer alone were immersed in the water-bath at the desired temperature. After allowing time for the tubes to come to the bath temperature, 3 ml. of the 2% β-lactoglobulin solution was added (making the final concentration 1% protein). After being heated for the desired interval of time the tubes were removed from the bath, cooled immediately in ice-water, stoppered and placed in the refrigerator. The samples were titrated after 48 hours using the *o*-iodosobenzoate–iodine titration procedure. It was assumed that after 48 hours complete oxidation of the activated sulfhydryl groups had occurred. The results of both experiments yielded a series of curves for the various temperatures when the time of heating was plotted against the *o*-iodosobenzoate titer (Fig. 5).

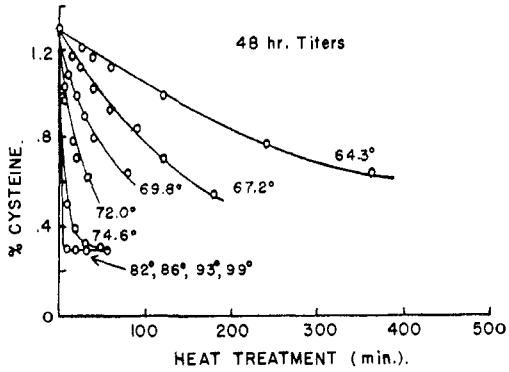

Fig. 5.—Influence of the temperature of heat treatment on the rate of the loss of the reducing power of β-lactoglobulin. The protein sols were heated at the indicated temperatures for variable lengths of time and allowed to stand for 48 hours before being titrated by the *o*-iodosobenzoate-iodine procedure.

In the various experiments reported in this paper

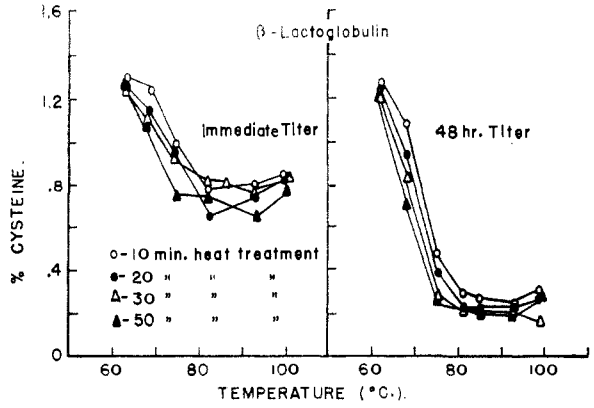

Fig. 4.—Change in reducing power of β-lactoglobulin during a 48-hour interval after heating as measured by the *o*-iodosobenzoate–iodine titration procedure. Titrations were made immediately and after standing for 48 hours on the protein sols which had been heated for the indicated times and temperatures.

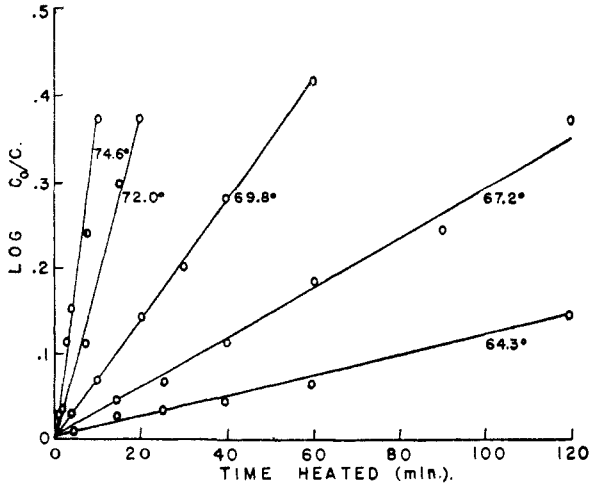

Fig. 6.—Compliance of loss in reducing power of heated β-lactoglobulin to first order kinetics. C_0 taken as 1.00% cysteine.

the minimum sulfhydryl content attainable by heat treatment and oxidation was 0.25–0.30% cysteine (Tables I and II and Figs. 2, 4 and 5). Thus sulfhydryl groups corresponding to only about 1.00% cysteine can be activated by heat to the extent that they are oxidized by oxygen.

Since the reaction does not follow zero order kinetics (Fig. 5), it was next sought to determine if the reaction is of the first order by plotting log C_0/C *vs.* time. In calculating log C_0/C, the value of C_0 was taken as 1.00% cysteine which represents the total amount of sulfhydryl that can be activated to oxidation by atmospheric oxygen. The plot of log C_0/C *vs.* time (Fig. 6) indicates satisfactory adherence to first order kinetics up to activation of about 60% of the groups. Above this point complications apparently occur with the result that first order kinetics no longer apply (not shown in Fig. 6).

The velocity constants for the reaction at each temperature are given in Table III and a plot of log k' *vs.* $1/T$ in Fig. 7, gives a linear relationship. The energy of activation, E, computed from the slope of this line ($E/2.303\ R$) is 80,600 calories per mole. Also in Table III are given calculated values for the thermodynamic functions ΔH^*, ΔF^* and ΔS^*, computed as outlined by Eyring and Stearn.[16] The values for these functions, as well as that for the energy of activation, are within the range reported for denaturation of other proteins as measured by other criteria.[16,17] Unfortunately, comparative data for denaturation of β-lactoglobulin as measured by properties other than sulfhydryl activity are not available; moreover, sulfhydryl activity has not been used in kinetic studies of the denaturation of other proteins. No fruitful comparison can be made between the rates of denaturation as measured by activation of sulfhydryl groups as reported in this study and by electrophoretic changes as observed by Briggs and Hull.[18] The latter workers differentiated the heat denaturation of β-lactoglobulin into two processes. The primary process, initiated only at temperatures above 65°, is not accompanied by any change in the electrophoretic mobility of the protein but does result in an approximately fourfold increase in particle weight. The secondary process, which takes place only after the first has occurred, results in a marked increase in mobility, is of the second order, and occurs at a maximum rate at 75–80°. It is possible that the activation of sulfhydryl groups parallels the primary denaturation observed by Briggs and Hull. However, no definite conclusion of this kind can be drawn since the data of these authors do not permit precise calculation of the kinetics and activation energy of the primary process. Certainly their secondary process does not appear to be related to changes in activity of sulfhydryl groups.

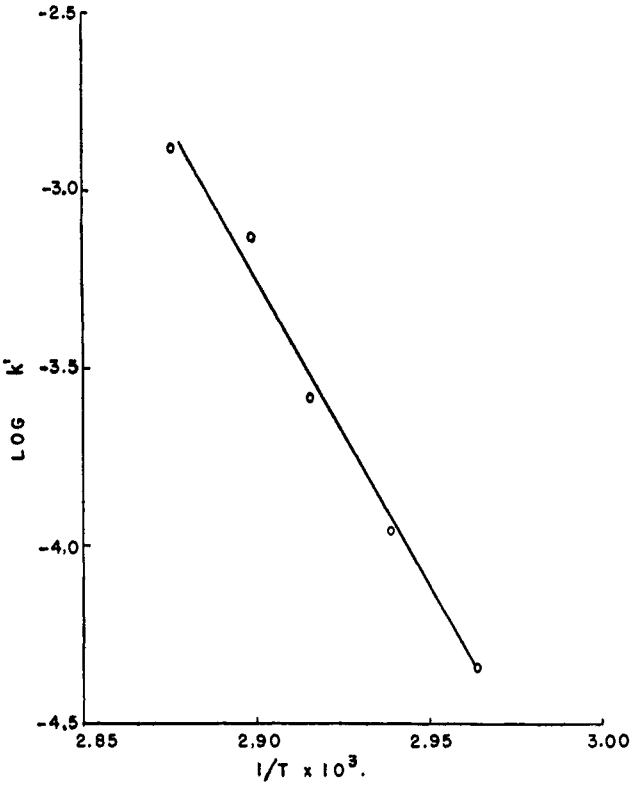

Fig. 7.—Relation between velocity constants and temperature. From the slopes of the line ($E/2.303\ R$), E is calculated to be 80,600 cal./mole.

TABLE III

VELOCITY CONSTANTS AND CALCULATED THERMODYNAMIC FUNCTIONS FOR HEAT DENATURATION OF β-LACTOGLOBULIN AS MEASURED BY LOSS IN TITRATABLE SULFHYDRYL GROUPS

Temp., °C.	k', sec.$^{-1}$ × 10^4	ΔH^*, cal./mole	ΔF^*, cal./mole	ΔS^*, cal./degree-mole
64.3	0.46	79,930	26,517	158.5
67.2	1.11	79,924	26,164	158.0
69.8	2.69	79,919	25,763	158.0
72.0	7.30	79,914	25,271	158.5
74.6	14.59	79,909	24,982	158.0

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(16) H. Eyring and A. E. Stearn, *Chem. Revs.*, **24**, 253 (1939).

(17) H. Neurath, J. P. Greenstein, F. W. Putnam and J. O. Erickson, *ibid.*, **34** 157 (1944).

(18) D. R. Briggs and R. Hull, THIS JOURNAL, **67**, 2007 (1945).