thohydroquinone (activities about 66% of that of 2-methyl-1,4-naphthoquinone) in the absence of oxygen. The corresponding glycosides of 3-methyl-1-naphthol gave complete response in minimum concentrations of 10 and 20 micrograms. The active 1-amino- and 1,4-diamino-derivatives of 2-methylnaphthalene were almost inactivated by conversion to the monoacetyl derivatives and these compounds as well as N-(2-methyl-1-naphthyl)-gluconamide and N-(1-amino-2-methyl-4-naphthyl)-succinamic acid were inapplicable.

3. An improved method for the preparation of 3-methyl-1-naphthol and 3-methyl-1-tetralone is described.

4. The structures of the esters of acetoxy-2-methylnaphthol and of the succinamic acid of 1,4-diamino-2-methylnaphthalene were established. From these orientation determinations structures of the new compounds examined for antihemorrhagic activity were ascertained. Substituents in the methyl of 2-methylnaphthalene lower the activity of the derivatives as compared with the parent quinone; 3-methyl-1,4-dihydroxyisoquinoline was inactive.

PEARL RIVER, N. Y. RECEIVED JULY 8, 1942

[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF HARVARD UNIVERSITY]

Heats of Organic Reactions. XIV. The Digestion of β-Lactoglobulin by Pepsin

BY GOTFRED HAUGAARD AND RICHARD M. ROBERTS

It is the aim of the experiments described here to apply calorimetry to the study of proteolytic processes. During the hydrolysis of a protein peptide bonds adjacent to many different types of side-chains are broken, and the peptides and amino acids formed have widely different ionization properties. The thermochemistry of the process is thus of such complexity that it is scarcely to be hoped that measurement of the net heat evolution would yield results of thermodynamic significance. We proposed, therefore, to use the calorimeter as an indicator, and to supplement the thermal measurements by simultaneous chemical determinations.

Acid or alkali have two drawbacks as hydrolytic catalysts for our purposes. The high temperature required for a reasonable rate of reaction is impractical for calorimetric work. Furthermore, acid and alkali are relatively unspecific in their attack of peptide bonds. Enzymatic catalysis eliminates both of these difficulties. The reaction can be carried out near room temperature. Proteolytic enzymes are specific in their action; each enzyme attacks only a few types of peptide bonds, so that a smaller number of processes is involved in the reaction.

Crystalline β-lactoglobulin was chosen as the substrate for the present work. This protein is easily prepared in a salt-free condition, contains no lipid or carbohydrate, and is homogeneous in the ultracentrifuge and electrophoresis cell.

Experimental Procedure

The enzyme selected was crystalline pepsin, which has a *p*H optimum around 1.5. In this case the buffering power of hydrochloric acid is sufficient to maintain constant *p*H. Complication of the heat evolution in an unknown manner by ionization effects, as would be the case for other enzymes requiring solutions buffered near neutrality, is thus avoided.

Enzyme and substrate solutions were made up in sufficient quantity for two simultaneous digestions to be made, one in the calorimeter, to measure heat evolution, and the other separately in a thermostat at the same temperature, using the same proportions of enzyme and substrate solutions in both digestions. Samples of the latter digest were removed at frequent intervals for determination of the following quantities: amino nitrogen, nitrogen not precipitable by trichloroacetic acid, and nitrogen dialyzable through cellophane.

Lactoglobulin.—Crystalline β-lactoglobulin was prepared from raw, skim milk by a modification of the original procedure suggested to one of the authors by Dr. A. H. Palmer.[1] After removal of the casein by coagulation at *p*H 4.5, the *p*H of the whey was readjusted to 6.0–7.0. The less soluble whey proteins are first precipitated by addition of solid ammonia sulfate to half saturation.[2] Solid ammonium sulfate was added to the filtrate from this first precipitate to 80–85% saturation. This second precipitate was redissolved in a minimum amount of water, adjusted to *p*H 6.0–6.5, and the subsequent dialysis and crystallization were carried out in the manner described in Palmer's article cited above.

The crystalline β-lactoglobulin so obtained was recrystallized as follows. The crystals were suspended in water and dissolved by addition of the minimum amount

(1) Palmer, *J. Biol. Chem.*, **104**, 351 (1934).

(2) See, in this connection, M. and S. P. L. Sörensen, *Compt. rend. trav. lab. Carlsberg. Sér. chim.*, **23**, 61 (1939).

of 0.1 *N* alkali, filtered and readjusted to *p*H 5.2 with 0.1 *N* hydrochloric acid. After seeding, the solution was allowed to stand at room temperature for three to four hours, and then left in the refrigerator for one to two days. The crystals were filtered on a Büchner filter, dried over calcium chloride, and then ground to a powder in a mortar. The powder was dried over phosphorus pentoxide, and was stored in a tightly stoppered bottle in the refrigerator. It is our experience that β-lactoglobulin, treated in this way, can be kept for years without losing its ability to crystallize, and upon dissolving in salt solution leaves behind only a trace of insoluble material.

To make solutions of β-lactoglobulin, the dry powder was dissolved in weak potassium chloride solution. The faintly cloudy solution was clarified by filtration. The filtrate was diluted so as to contain about 3 mg. N/cc. and be 0.15 *N* in electrolyte. Lactoglobulin solutions appear to keep well in the cold for at least two weeks, but in the present work the solutions were always used one to two days after preparation.

Preliminary work showed that β-lactoglobulin is made very much more digestible by pepsin at *p*H 1.5 by previous exposure to *p*H 11. It was found that denaturation of β-lactoglobulin occurs slowly at *p*H 9.5, and that the rate increases with the *p*H. The protein is completely insoluble at *p*H 5.2 after exposure to *p*H 11 for a few minutes. On the other hand, β-lactoglobulin appeared to be stable at fairly low acid *p*H. After exposure to *p*H 1.5 for four hours no precipitate formed upon titration to *p*H 5.2. We therefore assumed that the protein was unaltered by *p*H 1.5. Subsequent work proved that this assumption was false. Samples of the digest of native protein were adjusted to *p*H 6–7 and dialyzed as described below. After the prolonged dialysis the contents of the bags were brought to *p*H 5.2 and seeded with a few crystals of β-lactoglobulin. Some amorphous precipitate settled out on standing, but no sample showed any evidence of formation of new crystals. In order to decide whether the pepsin or the acid was responsible for destroying the ability of the protein to crystallize, a control experiment without the pepsin was carried out. A solution of native protein at *p*H 5.2 was brought to *p*H 1.5 with 0.27 *N* hydrochloric acid. Samples were removed at frequent intervals, starting at five minutes, were adjusted to *p*H 6–7 with 0.28 *N* potassium hydroxide, and dialyzed as before. A sample of the native protein solution at *p*H 5.2 was dialyzed under the same conditions. After dialyzing with frequent changes of water for ten days, all samples were brought to *p*H 5.2 and seeded. Only amorphous material was found in the samples which had been exposed to *p*H 1.5. The sample of native protein solution untreated with acid contained only β-lactoglobulin crystals. That the denaturation was not caused by the alkali during back titration of the acid-treated protein to *p*H 6–7 is proved by the fact that native β-lactoglobulin at *p*H 5.2 can be recrystallized without loss after titration with 0.28 *N* potassium hydroxide to *p*H 9.5.

Exposure to *p*H 1.5 for only five minutes, therefore, destroys the ability of lactoglobulin to crystallize. When the protein has been thus treated with acid, it must be regarded as a different substance, although its complete solubility at *p*H 5.2 makes it appear likely that less alteration of the molecule has occurred than in the case of the protein treated with alkali at *p*H 11.

In order to avoid circumlocution in what follows, "denatured protein" and "native protein" will refer to the previous history of the protein *before* adjusting it to *p*H 1.5 for peptic digestion.

Pepsin.—Crystalline pepsin was prepared from Cudahy Spongy Pepsin 1:10,000 and recrystallized once by the method outlined by Northrop.[3] The pepsin crystals were stored in the cold under saturated magnesium sulfate solution. About one-third of the weight of the crystalline paste was protein.

Pepsin solutions to be used in the digestions were made in the following way. The crystalline paste was suspended in water and dialyzed for two days against water acidified to *p*H 4.5 until most of the sulfate was removed. A measured quantity of potassium chloride solution was then added to the suspension, which was kept at 30–35° for an hour or two to hasten solution. The solution was filtered and diluted so as to be 0.15 *N* in potassium chloride and contain about 0.7 mg. N/cc. Pepsin solutions were stored in the cold under toluene and were always used within one day after preparation.

Determination of Amino Nitrogen.—The increase in amino nitrogen during the digestions was measured by the manometric method of Van Slyke.[4] The saturated solution of sodium nitrite used contained 10 g. of potassium iodide per liter.

Deamination of β-lactoglobulin for five minutes in the Van Slyke apparatus yields 25.5 equivalents of amino nitrogen per mole; in ninety minutes, 36.1 equivalents per mole are produced,[5] part of which is accounted for by the 29 lysine residues found by analysis. This large evolution of non-α-amino nitrogen in the time interval (three to four minutes) required for complete deamination of the α-amino groups leads to a high initial point for the digestion. Thus in order to obtain reproducible and comparable results the time of reaction in the Van Slyke apparatus must always be strictly the same at a given temperature. At the time these experiments were performed the authors were not fully aware of the fact that the lysine content of β-lactoglobulin is so large. It is possible that somewhat greater precision could have been obtained if longer reaction periods had been used, so that slight variations in time interval would give a less variable evolution of non-α-amino nitrogen.

Trichloroacetic Acid.—Of the many reagents used to precipitate proteins, trichloroacetic acid has the reputation of precipitating the least amount of polypeptide from a mixture of protein and its degradation products. We have tried several of the current recipes for using this reagent and found them unsatisfactory. The procedure we have used was finally standardized as follows. A given volume of a protein solution containing enough protein nitrogen to enable one to make a precise Kjeldahl analysis was mixed with an equal volume of a solution containing 20% by weight of trichloroacetic acid, both solutions being at room temperature. The mixture was allowed to stand at

(3) Northrop, "Crystalline Enzymes," Columbia Univ. Press, New York, N. Y., 1939, p. 129.

(4) Peters and Van Slyke, "Quantitative Clinical Chemistry," vol. II, p. 385 (1932).

(5) Cannan, Palmer and Kibrick, *J. Biol. Chem.*, **142**, 803 (1942).

room temperature for twelve to fifteen hours with frequent shaking. The shaking reduces the amount of non-protein nitrogen carried down by the precipitate. At the end of this period, the mixture is filtered and washed three times on the filter with small portions of 10% trichloroacetic acid. After filtration the filter paper containing the precipitate is placed in a Kjeldahl flask for determination of nitrogen. The success of this procedure is to be judged by the fact that both native and alkali-denatured β-lactoglobulin can be precipitated from solutions containing 0–80% of the total nitrogen in the form of peptic digestion products, without carrying down considerable amount of non-protein nitrogen, as shown in the case of denatured β-lactoglobulin in Table I.

TABLE I

DENATURED β-LACTOGLOBULIN

% of digestion products	Protein N per cc. found, mg.	Protein N per cc. calcd., mg.
0	2.46	2.46
18	1.98	1.97
36	1.49	1.48
56	1.03	0.99
77	0.54	.49

Further confirmation of the exactness of the trichloroacetic acid procedure is found in the excellent agreement between the trichloroacetic acid results and those from the dialysis experiments described below.

Dialysis Experiments.—The dialysis experiments made on samples of the digests in Runs A and B were performed in slightly different ways. In the case of Run B we wished to attempt to crystallize the unattacked protein, and for this reason could not use heat-treated protein for the dialysis.

Run A.—Twenty cc. of the heat-treated digest samples was dialyzed in Visking cellophane bags against 200 cc. of distilled water for ten days in the refrigerator. A little toluene was added to the dialyzate.

Run B.—Twenty-five cc. of the digest was brought to *p*H 6–7 with about 15 cc. of potassium hydroxide solution and dialyzed against 200 cc. of water for ten days as above.

At the end of ten days the dialyzates were removed and concentrated to about 50 cc. by vacuum distillation at 40°. Determinations of amino and total nitrogen were made on the concentrated solutions.

In order to measure the extent to which dialyzable nitrogen had been removed from the bags during the ten-day dialysis, and compare the undialyzable nitrogen with that precipitable by trichloroacetic acid, the dialysis was continued in the case of A for thirteen days and in the case of B for seven days longer, this time against frequently changed water. The dialyzates obtained during this period were evaporated on the steam-bath, and the total nitrogen was determined in each.

The Digestions.—A series of digestion experiments with both native and denatured lactoglobulin were carried out, using the same procedure in each case for heat evolution, trichloroacetic acid, and amino nitrogen determinations. The results of these experiments were consistent. All the digestions were carried out at 30 ± 0.1°. For the sake of brevity data from only the last pair, in which dialysis experiments were also performed, will be presented here.

Run A: Denatured Protein.—To 750 g. of β-lactoglobulin solution (*p*H 5.15, approx. 2.9 mg. N/cc.) was added 0.28 *N* potassium hydroxide with stirring until *p*H 11.10 was reached. As judged by completeness of precipitation at *p*H 5.2 upon back titration, denaturation is complete after standing a few minutes at *p*H 11. The solution was allowed to stand for one hour at room temperature. At the end of this time the *p*H was 10.85. 0.27 *N* hydrochloric acid and water were added until the final weight of the solution was 1390 g. and the *p*H was 1.52; 840 g. was placed in the larger compartment of the calorimeter; 500 g. was used for the simultaneous digestion and 50 g. for initial point determinations. This solution was 0.15 *N* in electrolyte.

To 35.0 cc. of pepsin stock solution (0.67 mg. N/cc.) was added 0.27 *N* hydrochloric acid, water, and 2 *N* potassium chloride solution to make a final weight of 134 g., with a *p*H 1.50, 0.15 *N* in electrolyte; 62 g. was placed in the smaller compartment of the calorimeter, 37 g. was used for the simultaneous digestion and 35 g. for initial point determinations.

Run B: Native Lactoglobulin.—To 749 g. of native β-lactoglobulin solution (*p*H 5.13, approx. 3.1 mg. N/cc.) 0.27 *N* hydrochloric acid, water, and 2 *N* potassium chloride solution were added in such quantity that the final weight was 1390 g., electrolyte normality 0.15, and *p*H 1.50. The same quantities as before were used for the calorimeter, concurrent digestion, and initial point.

To 95 g. of pepsin solution (0.784 mg. N/cc.) water and 0.27 *N* hydrochloric acid were added in such quantity that the final weight was 134 g., electrolyte normality 0.15, *p*H 1.48. The enzyme solution was divided into 62-g., 37-g., and 35-g. portions as in A.

Before making the concurrent digestion outside the calorimeter the enzyme and substrate solutions were allowed to come to the proper temperature in a thermostat, and were quickly mixed by pouring back and forth from one vessel to the other, then replaced in the thermostat. Sampling of the digest was done as follows. About 25 cc. was quickly removed, placed in an Erlenmeyer flask, stoppered tightly with a rubber stopper, and swirled in a water-bath at 80° for five minutes. Only a slight cloudiness resulted from this treatment. The sample was stored under toluene in the cold, and the several determinations were made as soon as possible. This method of killing the enzyme appears to cause no further hydrolysis of protein. We found no increase in amino nitrogen when lactoglobulin and pepsin were separately treated in this way.[6]

The Calorimeter.—The calorimeter and its operation have been described previously.[7] The calorimeter is divided into two compartments holding the liquids to be mixed; into one was placed 62 cc. of pepsin solution, and into the other 840 cc. of lactoglobulin solution. The calorimeter was brought to a low thermal head by electrical heating, and after waiting for thermal equilibrium to be reached, the enzyme and substrate were mixed and readings on the main thermel were made at frequent inter-

(6) L. Miller, *J. Biol. Chem.*, **109**, lxvi (1935), has found no further hydrolysis when peptic digests of lactalbumin were inactivated by heating five minutes at 80–85°.

(7) Conn, Kistiakowsky and Roberts, THIS JOURNAL, **62**, 1895 (1940); Conn, Gregg, Kistiakowsky and Roberts, *ibid.*, **63**, 2080 (1941).

vals over a period of three hours. Several non-stirring periods were interspersed through the run to determine the heat of stirring, which we found did not change appreciably during the calorimetric runs.

Blank runs were made in the calorimeter to determine whether native or denatured lactoglobulin or pepsin evolved heat when exposed to *p*H 1.5.

1. **Native Lactoglobulin.**—Native lactoglobulin was brought to *p*H 1.5, and 900 cc. was placed in the calorimeter; 0.75 mg. N/cc.

2. **Denatured Lactoglobulin.**—Native lactoglobulin was exposed to *p*H 11.1 for one hour, then brought to *p*H 1.5; 900 cc. was placed in the calorimeter; 0.76 mg. N/cc.

3. **Pepsin.**—900 cc. of pepsin solution at *p*H 1.5, 0.15 *N* in potassium chloride, was placed in the calorimeter; 0.024 mg. N/cc.

No heat evolution could be detected in any case. There was no increase in amino nitrogen in 1 or 2 after standing for two hours at *p*H 1.5, showing that no hydrolysis by acid occurred.

Heats of dilution of pepsin and lactoglobulin solutions used in the digestions were negligible.

Experimental Results

The experimental results of Runs A and B are compiled in Tables II and III. In Fig. 1 the experimental values for the increase in α-amino nitrogen are plotted against the increase in nitrogen not precipitable by trichloroacetic acid. The points lie on straight lines of different slopes. Thus as the digestion proceeds the increase in amino nitrogen is proportional to the increase in nitrogen not precipitable by trichloroacetic acid. The data of Tables II and III (except the dialysis results) are plotted in Figs. 2 and 3. The curves for amino nitrogen have been smoothed by taking values from the straight lines of Fig. 1.

TABLE II

RUN A: DENATURED β-LACTOGLOBULIN

Time, min.	Amino N, mg./cc.	N precip. by trichlor. acid, mg./cc.	Dialysis: Non-dialyzable N mg./cc.	Dialysis: Dialyzate Amino N / Total N	Dialysis: Inner solution Amino N / Total N	Heat, evolution, cal. per 902 cc. of digest.
0	0.056	1.39				
5.5	.127	0.77	0.84			6.99
15.25	.145	.69	.64	0.146	0.035	9.50
30	.149	.57	.52	.170		11.37
45	.158	.46	.43	.145		12.52
60	.159	.42	.43	.166		13.88
90	.174	.32	.30	.155		16.66
120	.178	.26	.24	.148		19.09
150	.190	.24	.21	.144		21.21
180	.191	.21				23.10
210						24.85
244	.196	.18	.20	.154		
			Average	.153		

TABLE III

RUN B: NATIVE β-LACTOGLOBULIN

Time, min.	Amino N, mg./cc.	N precip. by trichlor. acid, mg./cc.	Dialysis: Non-dialyzable N, mg./cc.	Dialysis: Dialyzate Amino N / Total N	Dialysis: Inner solution Amino N / Total N	Heat evolution cal. per 902 cc. of digest.
0	0.056	1.41				
5.5	.082	1.23				0.48
10						.77
14				0.197	0.0406	
15						1.02
16.75	.081	1.19	1.16			
30	.088	1.16	1.25	.229	.0422	1.73
45						2.52
60	.100	1.10	1.17	.211		3.09
90						4.21
97	.108	1.06	1.05	.212		4.43
120	.116	1.05				5.10
150						5.93
189	.111	0.99	1.06	.218	.0425	
195						7.02
480	.123	.95				
1243	.191	.71	0.75	.212		
2800	.227	.39	.42	.218		
9 days	.329	.086				
			Average	.214	.0418	

The total non-dialyzable nitrogen and the nitrogen precipitated by trichloroacetic acid were found to be identical in amount, as seen from Tables II and III. Further tests showed that the concentrated dialyzates gave no precipitate with trichloroacetic acid, and the amino nitrogen content of the solutions in the dialysis bags after exhaustive dialysis was the same as that of undigested protein.

Referring to Tables II and III one can see that the ratio of amino to total nitrogen in the dialyzates is unchanged as the digestion proceeds. The amino nitrogen content of the dialyzates is easily seen to be in agreement with the slopes of the lines in Fig. 1. Native and denatured lactoglobulin both yield 4.3% of the total nitrogen as amino nitrogen in the standard reaction time for α-amino determination. The amino nitrogen found in the dialyzates will therefore be the sum of the amino nitrogen originally available and the α-amino nitrogen formed by hydrolysis. On the other hand the slopes of the lines of Fig. 1 take into account only the increase in α-amino nitrogen due to hydrolysis. Hence

$$\text{Slope of line} = (\text{amino N/total N})_{\text{dial.}} - 0.04$$

Substituting the experimental values

A. slope = 0.11, (amino N/total N) dial. = 0.15
B. slope = 0.17, (amino N/total N) dial. = 0.21

one finds that this relation is satisfied for both runs.

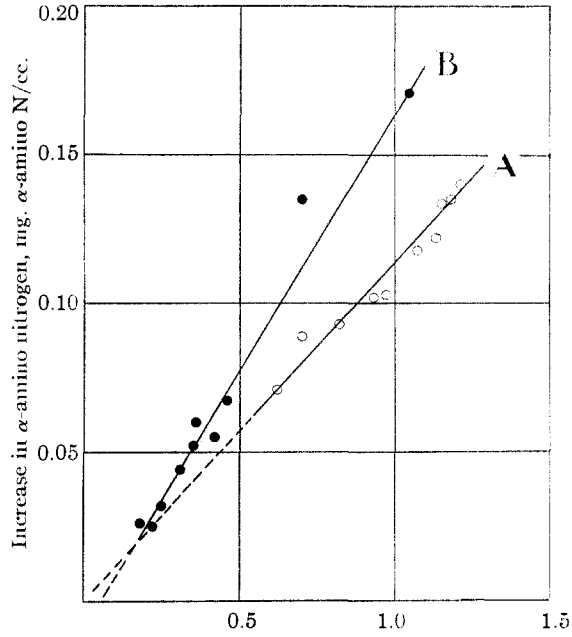

Fig. 1.—Increase in nitrogen not precipitable by trichloroacetic acid, mg. N/cc.

By applying the technique of solubility determinations it will probably be possible to improve the determinations of nitrogen precipitable by trichloroacetic acid, and the amino nitrogen determinations can, as previously mentioned, also be improved by using a longer reaction time. In this case the determinations in question may give figures of constitutional significance, especially when the specificity of the pepsin action is better known.

The amino nitrogen content of the dialyzates and of the inner solutions can be used to analyze the trichloroacetic acid and amino nitrogen data and confirm in a convincing way the existence of two fractions in the digests, whose amino nitrogen contents are independent of time. The trichloroacetic acid and amino nitrogen values in columns A and C of Table IV are taken from the curves of Fig. 1. The values in columns A and B are obtained by multiplying the precipitable nitrogen and the non-precipitable nitrogen present at a given time by the factors 0.04 and 0.153 given above for the amino nitrogen content of the inner solution and that of the dialyzate. The sums of

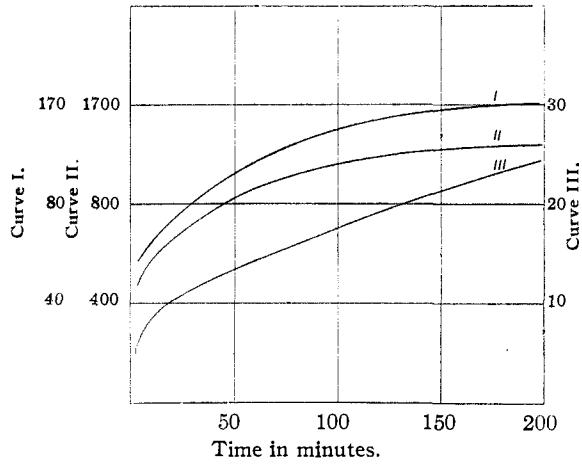

Fig. 2.—Curve I, total increase in α-amino nitrogen; II, total increase in nitrogen not precipitable by trichloroacetic acid; III, heat evolution in calories.

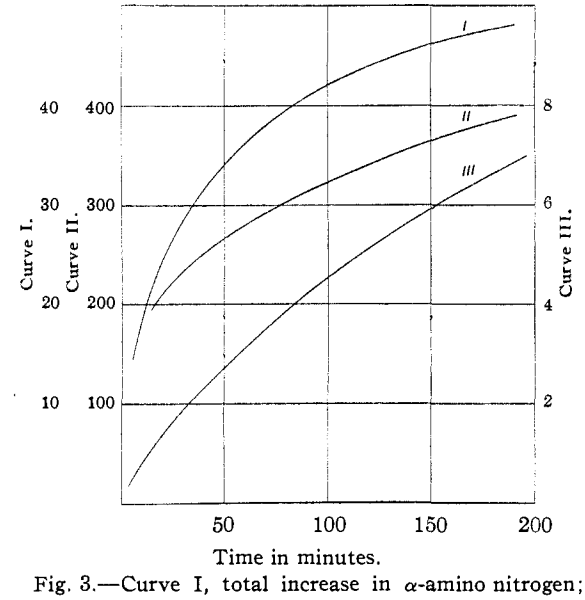

Fig. 3.—Curve I, total increase in α-amino nitrogen; II, total increase in nitrogen not precipitable by trichloroacetic acid; III, heat evolution in calories.

A and B in the next to the last column are to be compared with the experimental values for amino nitrogen in the last column. The excellent agreement is proof of the existence of two fractions in the digest: protein or protein-like material which is non-dialyzable and is precipitated by trichloroacetic acid, and a fraction consisting of much smaller particles which are completely dialyzable and are not precipitated by trichloroacetic acid. A similar analysis for Run B gave equally good results.

The ratio of amino to total nitrogen in the dialyzates is independent of time of digestion; and

TABLE IV

ANALYSIS OF DATA OF RUN A

Amino N/Total N in dialyzate, 0.153; amino N/Total N in lactoglobulin, 0.04.

Time, min.	[a] N precip. by trichlor. acid, mg./cc.	A 0.04 × a	[b] N not precip. by trichlor. acid, mg./cc.	B 0.153 × b	Calcd. amino N A + B, mg./cc.	C Exptl. amino N, mg./cc.
5	0.77	0.031	0.61	0.093	0.124	0.119
15	.69	28	.69	.106	.134	.134
30	.57	23	.81	.124	.147	.148
45	.46	18	.92	.142	.160	.159
60	.42	17	.96	.147	.164	.164
90	.32	13	1.06	.162	.175	.174
120	.26	10	1.12	.171	.181	.183
150	.24	10	1.14	.175	.185	.186
180	.21	8	1.17	.179	.187	.188
244	.18	7	1.20	.184	.191	.191

increase in amino nitrogen is proportional to increase in non-precipitable nitrogen (or to increase in dialyzable nitrogen). These facts suggest very strongly that when pepsin attacks the lactoglobulin molecule a definite number of fragments is produced at once, and these fragments are not further attacked by the enzyme. A similar conclusion was reached by Tiselius and Eriksson-Quensel after an electrophoretic study of peptic digests of egg albumin at the *p*H optimum.[8]

They found particles of unchanged size in the digest, but there was no evidence of particles intermediate in size between a molecular weight of 1000 and the original protein. That the nature of the digestion products is strongly influenced by the *p*H of digestion is shown by Petermann's ultracentrifugal analysis of the non-dialyzable fractions of peptic digests of beef serum pseudoglobulin between *p*H 2.7 and 4.5.[9] Besides particles unchanged in size, Petermann found particles which sedimented homogeneously which could be interpreted as halves and quarters of the original molecules; the distribution of nitrogen among the various fractions was a function of the *p*H. At *p*H 2.7 most of the nitrogen was in the unchanged and the dialyzable fractions; it thus appears likely that at the optimum *p*H there would be no evidence of products of intermediate size.

If our data are accepted as evidence of "explosion" of the lactoglobulin molecule, the number of bonds per molecule which can be hydrolyzed by pepsin can be determined from the slopes of the lines in Fig. 1. Taking the molecular weight of β-lactoglobulin as 39,000, and the value 0.11 for the slope of A, one finds that 46 peptide bonds per molecule have been broken in the case of denatured lactoglobulin, or 13.5% of the 341 peptide bonds in the molecule.[10] In the case of native lactoglobulin one finds that 71 peptide bonds per molecule, or 20.8% of the peptide bonds were split. It should be pointed out that although the native protein is attacked much more slowly, it is broken into smaller fragments. The average molecular weights of the split products are 870 for the denatured and 560 for the native protein. Particles deviating widely in molecular weight from the average may be present in the degradation products. We attempted to follow the evolution of free amino acids during the digestion by the ninhydrin–carbon dioxide method.[11] We found free amino acids to an extent of 1–2% of the total nitrogen in various digestions. Our results were not sufficiently accurate to detect an increase in the small amount of free amino acid with time of digestion. Blank runs showed that this hydrolytic product did not arise from exposure of pepsin or lactoglobulin to *p*H 1.5.

The calorimetric measurements were more accurate in the digestion of denatured protein. The heat evolution during the period of measurement was more than three times as large as in the digestion of native protein. The heat of stirring of the digests was about 0.04–0.05 cal./min. and could be measured during the reaction with a precision of only about ±5%. In Run A the error of measurement of the total heat evolution was ±2%, but in the digestion of native protein, where the rate of heating due to reaction was of the same order of magnitude as the heat of stirring, the measurement of the total heat evolution due to reaction was subject to very large error, estimated to be about ±13%.

In Fig. 4 the heat evolution is plotted against the increase in amino nitrogen after the same time of digestion. In both Runs A and B the heat evolved per peptide bond split increases markedly with time of digestion. From the slopes of the curves of Fig. 4 at various points one finds the following values:

Denatured protein	Native protein
2000 cal./mole peptide split at start	1300 cal./mole, at 15 min.
4000 cal./mole, at 90 min.	6000 cal./mole, at 190 min.
7000 cal./mole, at 150 min.	

(8) Tiselius and Eriksson-Quensel, *Biochem. J.*, **33**, 1752 (1939).

(9) Petermann, *J. Phys. Chem.*, **46**, 183 (1942).

(10) Hotchkiss, *J. Biol. Chem.*, **131**, 387 (1939).

(11) Van Slyke, Dillon, MacFadyen and Hamilton, *ibid.*, **141**, 627 (1941).

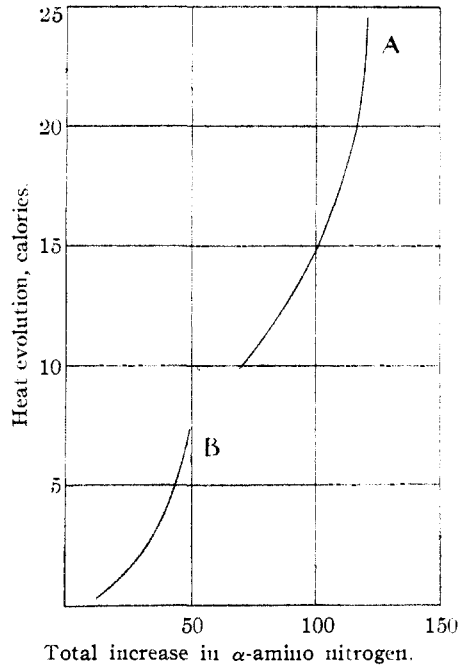

Fig. 4.

In case the degradation products were further hydrolyzed during the treatment with pepsin it could be expected that the heat per peptide linkage split would change as the process proceeded. The change would probably be small and the heat values consequently of the same order of magnitude. This is certainly not the case, and the most probable conclusion, therefore, is that not all the heat is due to the hydrolysis, but to processes we have not followed chemically. As we have shown that the degradation of β-lactoglobulin by pepsin is finished by the first attack, this secondary reaction—of unknown character—must be related to the breakdown products. Logically, of course, pepsin may attack other linkages of the degradation products than peptide bonds, but this is very unlikely. A rearrangement or an oxidation (or both) of some of the degradation products seems more probable.

Assuming that the heat value at the beginning of the experiment, where none or only a very small amount of the degradation products have reacted represents the true value of the heat of hydrolysis, we can use this value combined with the α-amino determinations to construct a heat–time curve which is related only to the degradation by pepsin of the lactoglobulin molecule. The difference between this curve and the actually found heat–time curve will thus represent the heat of the secondary reaction.

We have done this in the case of the run with denatured β-lactoglobulin using the value 2000 calories as representing the heat of hydrolysis. The set of curves are shown on Fig. 5, 1 is the actual heat curve, 2 the calculated heat curve, and 3 represents the heat evolution due to the secondary reaction.

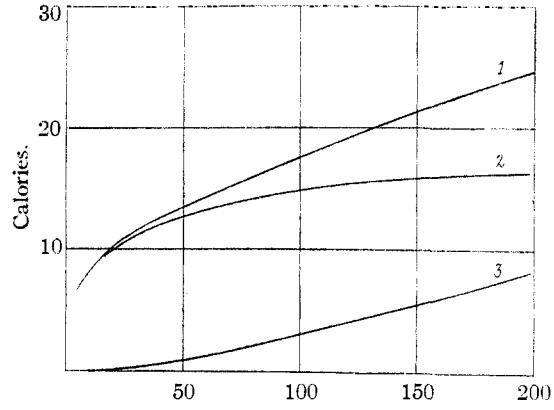

Fig. 5.

After an induction period at the beginning this third curve shows an increasing evolution of heat. The secondary reaction should thus be exothermic.

The lack of proportionality between heat evolution and extent of hydrolysis found in our experiments is reminiscent of dilatometric experiments of Linderström-Lang and Jacobsen on the digestion of native and denatured lactoglobulin and clupein by trypsin and chymotrypsin.[12]

The contraction per mole of peptide bond split was in the case of clupein, a protein considered of simple polypeptide chain structure and of low molecular weight, close to the normal value for peptides and was independent of time of digestion. In the digestion of native β-lactoglobulin, however, the contraction was initially about twice the normal value, and fell off slowly during the digestion. The contraction of heat denatured β-lactoglobulin was initially nearly normal, then rose to maximum almost twice the normal value, and thereafter decreased slowly. Linderström-Lang and Jacobsen believe that the abnormally large contraction may be due to the collapse of a "superstructure" originally present in the protein molecule.

In view of the experiments presented in this paper we should expect that the decreasing volume per peptide bond broken would be independent of

(12) Linderström-Lang and Jacobsen, *Compt. rend. trav. lab. Carlsberg, Sér. chim.*, **24**, 1 (1941).

the time as the degradation products are not further degradated. To prove this we have carried out a few dilatometric measurements using the technique described in detail elsewhere.[13,14]

The dilatometer used had two bulbs, the one (volume 45 cc.) containing at the beginning of the experiment the substrate, the other bulb (volume 8 cc.) containing the enzyme solution. Kerosene was filled in and the capillary tube inserted. The dilatometer was then immersed in water at 27 ± 0.005°. The concentrations of the enzyme and substrate were the same as in the digestion experiments presented on the foregoing pages. The β-lactoglobulin was alkali denatured. After the temperature equilibrium was reached the two solutions were mixed. The reading of the meniscus was continued for two hours. The results are tabulated in Table V.

TABLE V

Time, min.	Number of peptide bonds broken	Change in volume, cc.	Change in volume per peptide bond broken, cc.
5	0.000183	−0.00427	−23.4
10	215	528	24.6
15	241	586	24.4
20	260	630	24.2
30	286	698	24.4
40	305	748	24.6
50	318	783	24.6
75	344	841	24.5
100	360	867	24.1
125	373	877	23.5
		Average	−24.2

The contraction per peptide bond broken was found constant during the time of the experiment, which strongly confirms the results obtained by the trichloroacetic acid precipitations and α-amino nitrogen determinations (Fig. 1).

(13) Linderström-Lang and Lanz, *Compt. rend. trav. lab. Carlsberg, Sér. chim.*, **21**, 315 (1938).

(14) Linderström-Lang in Myrbäck and Baman, "Die Methoden der Fermentforschung," Leipzig, 1940.

In conclusion it may be said that it is difficult to suggest a plausible mechanism for the "explosive" degradation of lactoglobulin and other substrates by pepsin. As Tiselius and Eriksson-Quensel have pointed out, anything less than a simultaneous rupture of a great many peptide bonds would result in an almost continuous spectrum of products, and in our case the linear relationship of Fig. 1 would not hold. However, the notion that the enzyme molecule is able to attack many bonds in the substrate at once is difficult to believe. One is tempted to suggest the following hypothetical process: The splitting of a single peptide bond by the pepsin makes the "superstructure" or the "protein pattern" unstable, whereupon it decomposes.

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Summary

Native β-lactoglobulin and β-lactoglobulin denatured by alkali were digested by pepsin at *p*H 1.5. Heat evolution, nitrogen precipitable by trichloroacetic acid, increase in amino nitrogen, and dialyzable nitrogen were measured in the same digest as a function of time of digestion.

Precipitation with the trichloroacetic acid and dialysis through cellophane were found to be equivalent methods of fractionation of the total nitrogen in the digest.

Evidence for an "all-or-none" attack of lactoglobulin by pepsin is brought forward.

The heat evolution was not proportional to the extent of hydrolysis. It is suggested that digestion of lactoglobulin by pepsin is accompanied by an exothermic non-hydrolytic process.

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