[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, COLUMBIA UNIVERSITY, No. 411]

THE HYDROLYSIS OF COLLAGEN BY TRYPSIN[1]

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It is commonly stated in the literature of proteins[3] and of leather chemistry[4] that collagen is not attacked by trypsin unless it has been previously swollen with acids or alkalies, or shrunk in water at 70°, or treated with pepsin. Plimmer[5] advanced the theory that "those proteins which are resistant to the action of trypsin until they have been acted upon by pepsin will have all their units contained in the anhydride ring," and this has been used by Wilson in postulating a ring structure for collagen. In a private communication Plimmer states that "as far as I know there is no direct evidence that collagen has a ring structure. The statement is one of supposition."

A review of the literature reveals the fact that all statements as to the non-hydrolyzing action of trypsin on collagen date back to work by Kühne,[6] Ewald and Kühne,[7] and Ewald,[8] in 1887 and 1890. This was based on qualitative observation only, for very limited periods of time, and without regard to the significance of the reaction of the solution in contact with the substrate.

Object of Investigation

It was decided to investigate the action of trypsin on collagen, quantitatively, with special reference to the control of acidity, both in pre-treatment of the collagen and during contact with the trypsin. The object was to determine the effect, if any, of pre-treatment at different hydrogen-ion concentrations and to obtain more exact data on the optimum conditions of tryptic action on collagen.

[1] Presented before the Division of Leather Chemistry at the 64th meeting of the American Chemical Society, Pittsburgh, Sept. 4–8, 1922.

[2] Goldschmidt Fellow of Columbia University, 1921–1922; 1851 Exhibition Scholar of the University of Leeds, England.

[3] Compare Mann, "The Chemistry of the Proteids," The Macmillan Company, New York, **1906**, pp. 556, 557.

[4] Seymour-Jones, *Collegium* (London), **1**, 296 (1915). Rosenthal, *J. Am. Leather Chem. Assoc.*, **11**, 467 (1916). Wilson, *ibid.*, **12** 108 (1917). Wood, "Applied Chemistry Reports," Society of Chemical Industry, London, **2**, 368 (1917). Procter, "Principles of Leather Manufacture," E. and F. N. Spon, Ltd., London, **1922**, p. 138.

[5] Plimmer, "The Chemical Constitution of the Proteins," Longmans, Green and Co., London and New York, **1913**, 2nd ed., Part II, p. 11.

[6] Kühne, *Verh. d. Naturhist. Med. Ver. Heidelberg, N. S.*, **1**, 198 (1887).

[7] Ewald and Kühne, *ibid., N. S.*, **1**, 451 (1887).

[8] Ewald, *Z. Biol., N. S.*, 8, **26**, 1 (1890).

Materials Used.—"Standard Hide Powder, 1921"[9] was chosen as the source of collagen, both as the most convenient, pure form for quantitative work and in view of its importance in leather chemistry. This is prepared by grinding up hides which have been unhaired by liming and subsequently delimed by treatment with hydrochloric acid. It was rendered fat-free by extraction with chloroform. It contained collagen, a little elastin, and traces of keratins from the cell walls, with possibly a small amount of early degradation products of incipient hydrolysis. It was sifted before use through a 34-mesh sieve, and tests were made with both fine and coarse siftings.

Two commercial samples of trypsin were used, called "Pancreatin"[10] and "Trypsin,"[11] respectively.

The acidity was controlled by a series of buffer solutions, the hydrogen-ion concentration being determined electrometrically. The buffer mixtures covered a range of P_H + 1.9 to 9.9, and consisted of sodium citrate, potassium and sodium phosphates, and sodium borate, with hydrochloric acid or sodium hydroxide added as required.

General Method.—About 0.5 g. of hide powder was placed in a 10cc. centrifuge tube with a conical bottom, graduated in 0.1 cc.; 5 or 10 cc. of a buffer solution was added, together with 1 or 2 drops of toluene. The tubes were then corked and fastened to a shake machine rotating at 8 r. p. m. After a definite time, the tubes were removed and centrifuged for 20 minutes at 1200 "times gravity." The supernatant liquor was poured off and 5 or 10 cc. of fresh buffer solution added of the same hydrogen-ion concentration as that to be employed in the digestion. The hide powder was stirred in this, 1 or 2 drops of toluene added, and the shaking repeated to bring the hide powder to the new hydrogen-ion concentration. The tubes were centrifuged as before and the volume of hide powder noted.

The supernatant liquid was poured from the tubes, 5 or 10 cc. of a 1.0 or 0.5% trypsin solution, made up in a buffer solution and added, together with toluene as before, and the shaking repeated. The shaking was carried out in a Freas water thermostat maintained at 40.00°. The tubes were finally centrifuged for 20 minutes at the same speed, the volume of hide powder remaining undissolved was noted, and hence the amount digested by the trypsin determined.

A series of 16 centrifuge tubes was employed, all experiments being performed in duplicate and satisfactory checks obtained. Control tubes were run in every case, under identical conditions except that the trypsin was omitted. The pre-treatment was carried out first at 40.00°, but subsequently at room temperature in order to minimize hydrolysis. The tryptic digestions were always carried out at 40.00°.

The accuracy obtained in this method of measurement, which was adopted throughout the whole series of experiments, is limited entirely by that in reading the level in the tubes. With the centrifuge and the finely sifted hide powder it was possible to obtain a well defined boundary, and

[9] *Manufactured by the Standard Mfg. Co., Ridgway, Penna.*

[10] From Parke, Davis and Co.

[11] From Fairchild Brothers and Foster, New York City.

the percentage digestions so obtained are accurate to ±2. Considering the insoluble nature of the substrate, the method is probably the most accurate available, and is reasonably rapid. It is distinctly preferable to separation of the undigested hide powder by filtration and determination of the amount dissolved by an estimation of nitrogen in the filtrate; this is objectionable and inaccurate for two reasons: (1) it is difficult to filter the hide powder satisfactorily and to obtain a clear filtrate; (2) the digestion products are partially molecularly and partially colloidally dispersed, and any filtration will merely effect an arbitrary separation, dependent on the size of the filter pores. More particularly, part of the nitrogenous matter in solution will be sorbed by the undigested hide powder, and obtaining a true aliquot portion will be impossible.

Preliminary trials indicated that the hide powder was very considerably hydrolyzed by trypsin at 40.00°, although not greatly attacked at room temperature.

Effect of Pre-treatment.—The hide powder was shaken in buffer solutions of P_H 1.1 to 8.9 for 3 hours, then for a further 3 hours in buffer solutions of P_H 5.3 to 7.9, and finally subjected to the action of 0.5% trypsin solution at the same hydrogen-ion concentration for 6 hours at 40.00°. The results are summarized in Table I.

TABLE I

EFFECT OF PRE-TREATMENT ON HYDROLYSIS

Pre-treatment at P_H	Digestion at P_H	% Hydrolysis Trypsin	% Hydrolysis control	Pre-treatment at P_H	Digestion at P_H	% Hydrolysis Trypsin	% Hydrolysis control
3.0	5.3	69	7	1.1[a]	7.0	73	53
5.3		65	8	3.0		73	6
				5.3		75	18
3.0	5.9	81	20	7.0		80	32
5.3		89	34				
7.0		88	42	3.0	7.9	73	25
8.9		87	46	5.3		80	54
				7.0		81	72
				8.9		80	69

[a] Approximately 0.1 N HCl used.

It is clear from the above data that pre-treatment has no appreciable influence on the subsequent hydrolysis of the collagen by trypsin. It appears, however, to increase the ordinary hydrolysis at extreme acidity and with increasing alkalinity of pre-treatment.

Optimum Hydrogen-ion Concentration for Tryptic Hydrolysis.—It was evident from the foregoing that tryptic hydrolysis was greatest in the region around P_H 5.9. Hence a series of experiments was made at hydrogen-ion concentrations between P_H 5.3 and 7.0, at intervals of 0.2 Sörensen unit. The hide powder was shaken for 3 hours at the desired hydrogen-ion concentration, and then digested at the same concentration for 15 minutes with a 0.05% trypsin solution.

The optimum range was from P_H 5.7 to 6.1, the logarithmic mean of which is 5.87. Consequently, the optimum hydrogen-ion concentration for the reaction may be taken as P_H 5.9.

Time Effect and Velocity Constant.—The hide powder was shaken for 3 hours at P_H 5.9, centrifuged and measured, then digested with 0.5% trypsin solution of the same hydrogen-ion concentration at 40.00° for periods of from 5 minutes to 6 hours. Immediately on removal from the thermostat the tubes were centrifuged and measured. The results are given in Table II and Fig. 1.

Table II

Variation of Hydrolysis with Time

Time	% Hydrolysis			Time	% Hydrolysis		
Minutes	Trypsin	Control	k	Minutes	Trypsin	Control	k
5	69	0	0.102	60	78	10	0.011
10	68	0	.050	90	75	14	.007
15	72	4	.037	120	80	13	.006
20	75	1	.030	360	89	34	.003
30	74	0	.020				

The curve so obtained is similar to that commonly found for the action of trypsin on proteins. From the data, the velocity constant, k, was obtained by means of the unimolecular reaction formula, using natural loga-

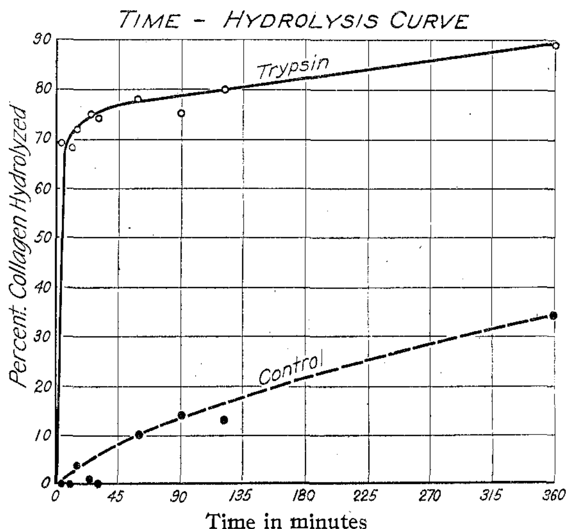

Fig. 1.—Time-hydrolysis curve.

rithms, and is inserted in the table. This "constant" steadily decreases with time, much in the same ratio as in a case cited by Bayliss[12] for the action of trypsin. This may be accounted for by (a) the disappearance or

[12] Bayliss, "The Nature of Enzyme Action," Longmans, Green and Co., New York, 4th ed., **1919**, p. 80.

destruction of the enzyme, (b) the effect of the hydrolysis products in reversing the reaction or destroying the enzyme, and (c) removal of the enzyme by its combination with the substrate or certain groups thereof.

Concentration of Enzyme.—The concentrations of trypsin employed in the foregoing experiments were high, in order to yield a definite and marked result. In the next set of experiments the concentration of the enzyme was varied. The procedure was as before, digestion being carried out at P_H 5.9 and 40.00° for 30 minutes. The results are given in Table III and Fig. 2.

Table III

Trypsin Concentration and Hydrolysis; Fine Particles

Concn. of trypsin, mg. per liter...	492.00	123.00	12.30	0.00 (control)
% Hydrolysis..................	74.	58.	26.	7.

There appears to be a limiting value at a trypsin concentration of about 10 mg. per liter, below which the ordinary hydrolysis is equal to that engendered by the enzyme.

A measurement of the hydrogen-ion concentration of the residual liquid of the above gave —P_H 5.8, showing that it had not been appreciably changed by the reaction products of the hydrolysis.

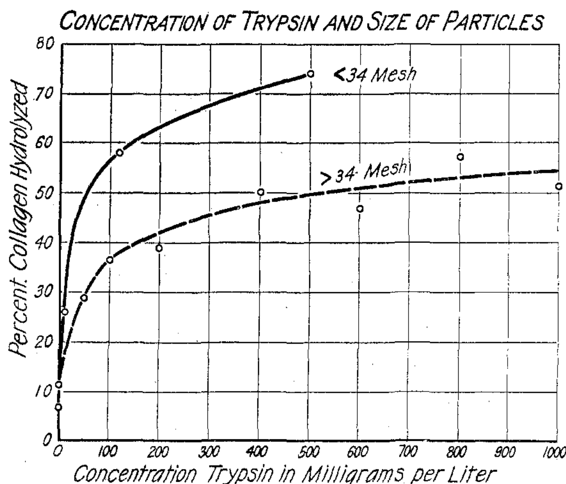

Fig. 2.—Concentration of trypsin and size of particles.

Size of Substrate Particles.—The hide powder employed in the preceding experiments consisted of that portion of "1921 Standard Hide Powder" which passed through a 34-mesh sieve. The coarser particles retained by the sieve were next used. Hydrolysis was carried out as before, using 0.5 g. of hide powder, at P_H 5.9, with various concentrations of trypsin, for 30 minutes at 40.00°. The results are given in Table IV and Fig. 2.

Table IV

Trypsin Concentration and Hydrolysis; Coarse Particles

Concn. of trypsin, mg. per liter	1000.	800.	600.	400.	200.	100.	50.	0.0
% Hydrolysis	51	57	47	50	39	36.5	29.	11.5

The larger and less uniform size of the particles of hide powder not only made it difficult to obtain an even level in the centrifuge tube but also offered a varying surface for the enzyme action, and made the results somewhat irregular. The lessened substrate surface naturally leads to a lower rate of hydrolysis. The limiting concentration of enzyme appeared to be of the same order of magnitude as with the fine hide powder.

Completeness of the Hydrolysis.—In none of the foregoing had 100% hydrolysis been obtained with the trypsin. An attempt was, therefore, made to bring the hydrolysis to completion by removing the reaction products from contact with the substrate.

Table V

Completeness of Hydrolysis

Time, min.	20	40	60	80
% Hydrolysis, trypsin	67	72	74	76
control	4	7.5	10	18.5

Half a gram of fine hide powder was placed in each of a series of tubes and shaken with 5 cc. of a phosphate buffer (P_H 5.9), as before, for 3 hours at room temperature. The tubes were then centrifuged and the volumes of hide powder measured. The supernatant liquor was poured off. To half the tubes 5 cc. of a 0.1% trypsin solution (made up in the same buffer) was added, and to the other half 5 cc. of the same buffer. These were shaken for 20 minutes in the thermostat at 40.00°, centrifuged and measured. The liquid was poured off, fresh trypsin and buffer solutions added, and the process repeated, 4 times in all. The experiment was stopped after 80 minutes, owing to the increasing hydrolysis in the control. The results are shown in Table V and Fig. 3.

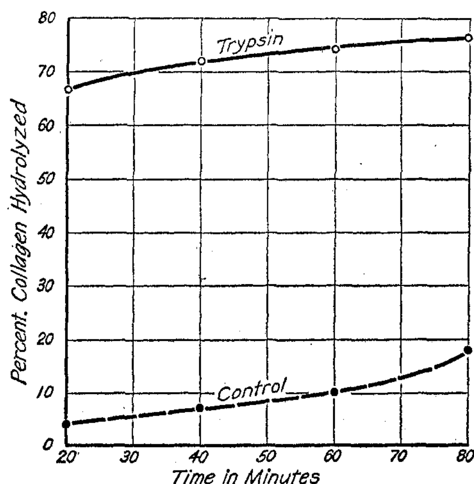

Fig. 3.—Residue digestion, with renewal of trypsin each 20 minutes.

The curve of trypsin hydrolysis is slightly concave to the time axis, and hence, probably the reaction never proceeds to completion, though it is not

possible to test this, since increasing the time greatly increases the hydrolysis in the control.

This result agrees with the work of Andersen,[13] who found it to be practically impossible to obtain complete hydrolysis of proteins by enzymes *in vitro,* although Abderhalden and Rona[14] claimed to have obtained complete hydrolysis of meat protein by digestion for 3 to 4 weeks with pepsin, trypsin, and erepsin, at 37°.

Reversibility of the Reaction.—Since we have to deal with an insoluble substrate, it is obvious that hydrolysis due to the trypsin can occur only when the trypsin is in contact with the hide powder. Now, the hide powder is swollen and contains water into which the colloidally dispersed trypsin can slowly diffuse. This diffusion, however, is extremely slow, as is shown by the difference between the effects with fine and coarse hide powder, and the slowness of the effect on skin. Hence, the action is probably almost entirely at the surface of the hide powder.

If, in Fig. 1, in place of calling the ordinate "percentage of collagen hydrolyzed" we name it "concentration of collagen" (since all action takes place in the collagen phase) and reverse the figures (that is, 100% hydrolysis = 0% of collagen), we then have the usual reversible equilibrium curve. In view of the surface action this may not be wholly justifiable, but it is here the only means possible. The reverse curve, that of the formation of collagen from hydrolysis products, cannot of course be obtained. Yet the curve shows that there is a perceptible though very slight reversibility, as would be expected, since the curve approaches but does not touch the 0% collagen line. The question is still further complicated by the fact that we have, as with all proteins, not a simple hydrolysis, but a whole series, from collagen through gelatin and intermediate stages down to amino acids. The trypsin curve, therefore, represents the whole series of actions, while the control curve merely covers the change from collagen to gelatin.

The general resemblance of the curve of trypsin with hide powder (Fig. 1) to those obtained with trypsin and soluble proteins—soluble in so far that they are probably colloidally dispersed—leads one to suspect that, since with trypsin-collagen the main action is undoubtedly at the surface, the action with trypsin-soluble protein is probably also at the surface of the colloidally dispersed protein. The trypsin itself is also in all likelihood colloidally dispersed.

Practical Applications.—In leather manufacture skins are treated in trypsin solutions, whereby the elastic fibres (elastin) are removed from the pelt, leaving the collagen fibers apparently unattacked. This process is known as *bating*. The skin at the same time "falls," that is becomes flaccid, this action being chiefly due to the acidity of the liquor. Strips of

[13] Andersen, *Biochem. Z.*, **70**, 344 (1915).

[14] Abderhalden and Rona, *Z. physiol. Chem.*, **67**, 411 (1910).

guinea-pig skin, treated for several hours at 40.00° in trypsin solutions of 0.1 to 0.4%, showed considerable loss of collagen. J. A. Wilson and A. F. Gallun[15] have also shown that calfskin, after a preliminary passive period, may be almost completely hydrolyzed with trypsin. It may be remarked that no such concentrations of trypsin as those herein indicated are obtained in using the ordinary commercial bates in practice.

Summary

A study has been made of the hydrolysis of collagen with trypsin, by controlling the acidity of the pre-treatment and digestion, and examining the effects of varying the time of digestion, concentration of enzyme, and size of collagen particles. The optimum hydrion concentration for the hydrolysis is found to be at P_H 5.9. Pre-treatment of the collagen in solutions of various degrees of acidity does not influence the subsequent tryptic digestion. The speed of hydrolysis increases as the size of the substrate particles diminishes, the action taking place probably at the surface of the particles. Hydrolysis increases with increasing concentration of trypsin, but never reaches completion under the limits of experimental conditions. The reversibility of the reaction is discussed, and the similarity between the action of trypsin on insoluble and soluble substrates is demonstrated.

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[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF THE UNIVERSITY OF CALIFORNIA]

THE BENZIL REARRANGEMENT. III

BY ARTHUR LACHMAN

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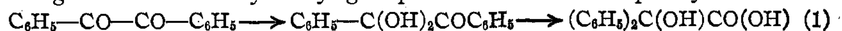
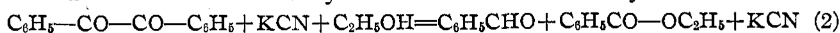
In the preceding papers[1] of this series, it has been shown that the conversion of benzil into benzilic acid may be regarded as an addition of water, or of alkali hydroxide, to one of the carbonyl groups, followed by an interchange of one of the hydroxyl groups thus formed with phenyl.



$$C_6H_5—CO—CO—C_6H_5 \longrightarrow C_6H_5—C(OH)_2COC_6H_5 \longrightarrow (C_6H_5)_2C(OH)CO(OH) \quad (1)$$

Intramolecular rearrangement (metakliny) of this sort, which also involves simultaneous oxidation and reduction, is always accompanied, to a greater or less extent, by a rupture of the molecule.

The present paper deals with the action of potassium cyanide on benzil. This interesting reaction was first reported by Jourdan.[2] Jourdan rubbed 20 g. of benzil in a mortar with 10 cc. of alcohol and 1 g. of potassium cyanide. The benzil disappeared completely in a short time; it was converted into a mixture of ethyl benzoate and benzaldehyde.



$$C_6H_5—CO—CO—C_6H_5+KCN+C_2H_5OH=C_6H_5CHO+C_6H_5CO—OC_2H_5+KCN \quad (2)$$

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[15] Wilson and Gallun, *J. Ind. Eng. Chem.*, **14**, 834 (1922).

[1] Lachman, THIS JOURNAL, **44**, 336 (1922); **45**, 1509 (1923).

[2] Jourdan, *Ber.*, **16**, 659 (1883).