by the length and type of the carbon chains of the entering groups where the molecular weight is constant.

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A COMPARISON BETWEEN THE CHEMICAL AND PHYSIOLOGICAL CHARACTERISTICS OF PEPSIN AND RENNIN

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This investigation was carried out for the purpose of obtaining more specific knowledge regarding pepsin and rennin. In dealing with these two enzymes, whose physiological characteristics have been known and utilized for many years, subjects must unavoidably be touched which have been general knowledge for a long time. Such findings should merely be considered as confirmative evidence.

The glandular layer of the fundus portion of hog stomachs forms the basic material for all manufactured pepsin, while rennin is obtained from the mucus lining of the fourth stomach of young calves. These raw materials were examined first. The mucus linings, whether from hogs or calves, were dissected out and carefully freed from fat and muscular tissue, finely minced and desiccated in a vacuum at 35° to 40° to constant weight. Analyses of the desiccated substances for the material soluble in petroleum ether, moisture, ash, total phosphoric acid and total nitrogen were made; and the proteolytic, milk-curdling and hemostatic activities were also determined. The total chlorine was determined on the fresh minced linings and the inorganic chlorine on the ash. The difference between the two represents the amount of free hydrochloric acid and organic chlorine present in the normal fresh linings. The proteolytic activity was determined on all samples according to the assay method of the U. S. Pharmacopeia and the hemostatic properties estimated on oxalated beef plasma. The milk-curdling tests were carried out on fresh certified milk, on pasteurized milk and on milk acidified with lactic acid to correspond to an acidity of 0.2% of lactic acid with phenolphthalein as indicator.

The calf mucosa is quite tender, which may account for the considerable number of stomach ulcers encountered. In some cases the ulceration is superficial, although distinctly outlined; in others the muscular coat of the stomach is quite corroded.

Both hog and calf stomach linings possess considerable hemostatic properties. The importance of the ability to check capillary oozing

promptly in an organ such as the stomach is obvious. Desiccated hog linings clot 1,000 times their own weight of oxalated blood in 55 seconds, while the calf linings will do the same work in 80 seconds. The cephalin fraction from the hog mucosa constitutes about 2.3% of the dried substance, while in the calf linings it is a little less. The proteolytic power of dry hog stomach linings varies from 1:750 up to 1:2,500. The milk-curdling activities are insignificant. The restricted action of pepsin, whether in the crude or purified state, on sweet milk is a consequence of this enzyme's requirement for a certain amount of acid for acting efficiently. The milk-curdling activity of pepsin consequently depends on the acidity of the milk. It should be recalled that the reaction of cow's milk is always on the acid side of neutrality, due to the presence of acid phosphates, citrates and casein. This slight acidity is sufficient to allow the pepsin to exercise some degree of hydrolysis and produce clotting, when present in certain concentrations. As the acidity of the milk increases, the proteolytic power of pepsin and consequently its milk-curdling activity become more pronounced. When the acidity reaches the equivalent of 0.2% lactic acid, the pepsin is able to exercise full proteolytic activity and its clotting ability becomes entirely dependent on the digestive power. For instance, the most active pepsin reported in this paper, showing a proteolytic power of 1:25,000, clotted sweet milk in proportions of 1:30,000 in $1^3/_4$ minutes. In dilutions of 1:60,000 no clotting occurred. After the milk was acidulated to correspond to 0.2% of lactic acid, the same pepsin curdled in proportions of 1:492,000 in $5^3/_4$ minutes. It has been our experience, however, that 2 solutions of the same sample of pepsin, but of different strength, do not give concordant results even with acidified milk. As is well known, the clotting time of rennin on sweet milk is directly proportionate to the concentration of the enzyme. No such time law exists for the action of pepsin on milk. It follows, therefore, that stomachs of adult hogs do not contain rennin and that any clotting properties of the gastric juice are due solely to pepsin.

Straight desiccated linings of calf stomachs curdle milk in proportions ranging from 1:2,000 to 1:5,000, depending on the acidity of the stomach. The average digestive power is about 1:350. By adding hydrochloric acid to the fresh calf mucosa before drying, the rennetic activity is greatly increased. For instance, a sample of straight dried linings showed a milk-curdling yower of 1:4,500 and digestive power of 1:350. The addition of 0.18% and 0.36% of hydrochloric acid increases the milk-curdling power to 1:49,000 and 1:138,000, respectively. The proteolytic power remained unchanged. Hammarsten showed long ago that the rennetic enzyme is produced and stored in some inactive form and activated by contact with acid in the stomach. There is, however, another important factor present, namely, the pepsin. It will be shown later that active

rennin is a product of protein decomposition and the possibility is not excluded that pepsin, which is always present in the calf mucosa, aids the hydrochloric acid in the hydrolyzing process.

The analytical data for the desiccated, fat-free linings, both from calves and hogs, were quite similar, the main difference being in the higher chlorine content, both organic and inorganic, of the hog stomachs.

The purified pepsin preparations were made from the minced fresh linings of hog stomachs by digesting them with acidulated water in suitable proportions. The resulting golden-yellow liquid was filtered. In the dry form this constitutes crude pepsin, possessing a proteolytic power of from 1:2000 to 1:4000, depending on the quality of the raw material. After the removal of mucin and other inert proteins by proper means, a 1:10,000 pepsin is readily produced. The 1:15,000 and 1:25,000 test pepsins were prepared by fractional precipitation and dialyzation of the mucin-free material.

A number of samples of rennin were produced by the well-known salting-out process. The highest-testing product made by this method contained 24% of sodium chloride and considerable insoluble protein material; it showed a milk-curdling power of 1:660,000 in 10 minutes and a proteolytic power of 1:1975. The sample reported in the tabulation is representative of the average run of rennins made by this process.

Attempts to purify the salted-out rennins by means of dialysis in neutral distilled water, in tap water or in water acidulated with hydrochloric acid, invariably proved fatal to the milk-curdling enzyme. The samples of rennins which were submitted to dialysis lost from 70 to 95% in weight and showed corresponding or even greater decreases in milk-curdling activity. It is only a small percentage of active rennin which can be recovered from the dialyzing liquid. The major portion is hydrolyzed during the process and cannot be restored. The proteolytic power of the dialyzed products was considerably higher than that of the original samples. It should be borne in mind that we are not here dealing with pure rennins, but merely with salted-out extracts of calves' mucosa, which contain both rennin and pepsin in varying amounts. The rennetic enzyme dialyzes freely through parchment paper, while pepsin does not, or at most only to a limited extent. The dialyzing process simply changes the relative proportion of the two enzymes.

Further experimental work developed that rennin is precipitated from acid solution on careful neutralization just before neutrality to litmus is reached. The precipitate is inert, or very nearly so, as far as milk-curdling properties are concerned, but the activity is restored when sufficient acid is added to render the protein again water-soluble. Pepsin is not precipitated by this method. Acid pepsin solutions also yield albumin precipitates as neutrality is approached. The preoteolytic power of these pre-

cipitates runs about the same, whether they are derived from rennin or pepsin solutions, showing only that a certain amount of pepsin is carried down with this class of proteins. If the main portion of free pepsin were precipitated by this neutralization method, the pepsin solution would yield precipitates many times stronger in proteolytic power than the rennin solution. This is not the case. The following method was adopted for the separation of the rennin.

The vacuum-dried, de-fatted and powdered mucus linings from calves' stomachs were macerated with 20 volumes of distilled water. The mixture was then acidulated with hydrochloric acid and again macerated for a few hours. The liquid portion was separated by centrifugation and filtration. Sodium bicarbonate solution was added cautiously until the reaction of the liquid was just faintly acid to litmus. The rennin separates slowly as colloidal masses, which settle with difficulty. Precipitation was facilitated by adding 2 volumes of distilled water and placing the mixture in the refrigerator overnight. The liquid was removed by decantation and the precipitate centrifuged. It was washed in the centrifuge tubes with several portions of distilled water until the washings were free from chlorine. The white precipitate, which still contained besides rennin considerable quantities of mucin and other mechanically carried-down impurities, was suspended in distilled water and sufficient dil. hydrochloric acid added to bring the reaction of the liquid to neutrality using methyl orange as indicator (golden-orange). By this procedure the rennin goes into solution, leaving most of the impurities in suspension. The turbid, slimy liquid was centrifuged at high speed until the mucin, etc., had separated and the supernatant liquid was clear. The precipitation and washing process was repeated until no further increase in rennetic activity was obtained. It was, therefore, concluded that the enzyme was free from impurities removable by this method and the clear liquid finally obtained was desiccated in a vacuum over sulfuric acid at room temperature. The finished product appeared as transparent, pale straw-colored scales.

About a dozen lots of rennin were made according to this method. The sample analyzed and reported in the table was a composite of the 2 preparations, giving the highest tests. No specific relation seems to exist between the milk-curdling and the proteolytic activity of these samples. One sample tested 1:2,310,000 in milk-curdling power and only 1:500 in digestive power; a second sample, 1:2,214,000 and 1:1,100; a third, 1:1,540,000 and 1:2,500; and a fourth, 1:960,000 and 1:1,000, respectively. Proteolytic or peptic activity evidently is not a part of the true physiological characteristics of the milk-curdling enzyme.

Slightly acidulated solutions of the purified enzymes gave the biuret and other protein reactions. A marked distinction was shown when the liquids were boiled. All the pepsin solutions became turbid and heavy flocculent precipitates separated. In the case of the high-testing rennins, which were nearly free from pepsin, the boiled solutions remained as clear and bright as the control. Both enzymes lost all activity on boiling.

This investigation covered a period of several years, during which the technique of preparation of the enzymes has been improved to such a point that we are now unable to raise the activity any further with the

PROPERTIES OF PEPSIN AND RENNIN

	Material sol. in pet. ether %	Moisture %	Ash %	Total N %	Total phos. acid as P_2O_5 %	Free and org. combined chlorine as Cl %	Inorganic chlorine as NaCl %	Proteolytic power	Milk Curdling Power at 40°			
									Certified sweet milk. Acidity corresponding to 0.15% lactic acid. Phenolphthalein as indicator	Commercial pasteurized milk. Acidity corresponding to 0.13% lactic acid. Phenolphthalein as indicator	Pasteurized milk acidified with lactic acid to correspond to 0.2% lactic acid. Phenolphthalein as indicator	Blood-curdling power on oxalated beef plasma containing 0.1% sodium oxalate at 37.5°
Glandular layer (fundus portion) of hog stomachs, desiccated. Moisture in fresh linings, 83.4%.	7.50	2.60	5.95	12.38	2.70	1.30	0.70	1:2500		1:15 in 4½ min. 1:30 no clot	1:230 in 5¾ min.	1:1000 in 55 sec.
Crude pepsin made by digesting linings with water acidulated with HCl, settling, filtering and desiccating.	none	3.25	4.80	12.87	1.55	4.10	1.80	1:4000		1:7500 in 3¾ min. 1:15,000 no clot	1:70,500 in 5¾ min.	none
Purified pepsin	none	5.00	2.10	14.48	1.52	0.68	trace	1:10,000		1:10,000 in 3¼ min. 1:20,000 no clot	1:192,000 in 5¾	none
Purified pepsin	none	2.10	1.35	14.90	1.57	0.10	none	1:15,000		1:10,500 in 2¼ min. 1:20,000 no clot	1:288,000 in 5¾ min.	none
Purified pepsin	none	3.20	1.75	14.90	1.48	0.15	none	1:25,000	1:30,000 in 1¾ min. 1:60,000 no clot	1:20,000 in 2 min. 1:40,000 no clot	1:492,000 in 5¾ min.	none
Desiccated mucosa from 4th stomach of calves 2 – 3 months old. Moisture in fresh linings, 86.8%.	5.0	3.80	6.20	12.47	2.70	0.42	0.10	1:350	1:3000 in 10 min.	1:2600 in 13½	1:24,000 in 5¾ min.	1:1000 in 80 sec.
Salted-out rennin.	...	2.40	24.70	11.30	0.51	2.25	23.25	1:1750	1:591,000 in 10 min.	1:549,000 in 13½ min.	1:882,000 in 5¾ min.	none
Purified rennin	...	6.50	1.05	14.00	0.69	1.75	none	1:600	1:2,310,000 in 10 min.	1:2,160,000 in 13½ min.	1:3,900,000 in 5¾ min.	none

material at our disposal. While it is realized that the purified samples of pepsin and rennin described in this paper are not absolutely pure enzymes, it is very evident that both pepsin and rennin are proteins. Our failure to produce a pepsin-free rennin undoubtedly is due in part to the fundamental characteristics of pepsin, but principally to the present very imperfect knowledge of the chemistry of proteins and to the primitive methods available for their separation or isolation.

Pepsin is coagulated by heat and is colloidal in nature. The main bulk of pepsin does not dialyze through parchment membranes and is recoverable. It is resistant to acids and exercises its maximum hydrolyzing properties in the presence of hydrochloric acid. Its proteolytic power and milk-curdling activity are identical. The slight amount of organic chlorine found in the samples giving the highest tests is not a natural constituent of the enzyme, but apparently is composed of impurities which have resisted the precipitation and dialyzing process. Pepsin is completely soluble both in neutral and acidulated water. In the crude form, when associated with other proteins and their decomposition products, pepsin is carried down mechanically with the precipitated protein fractions when the solution is saturated with sodium chloride. This apparently is evidence of its characteristic affinity for protein, because purified pepsin is clearly and completely soluble in saturated sodium chloride solution. Its keeping qualities in the dry form are excellent.

Physiologically active rennin is formed by the action of hydrochloric acid, and possibly pepsin, on some native protein secreted by the gastric mucosa of suckling calves. Its solubility in water and also its milk-curdling activity depend on the combination of a certain specific amount of hydrochloric acid with the protein. The union of the acid and the protein is rather loose, and dissociation takes place gradually even in the powdered dry form. This process renders the enzyme partly soluble in water, with proportional reduction in curdling power. By replacing the lost acid, the enzyme is brought back into solution and its clotting power restored. It is this dissociation which is responsible for the usual statement that rennin preparations deteriorate rapidly.

In acid solution rennin is not coagulated by boiling, but its specific physiological property is destroyed. Rennin dialyzes through parchment membranes and is hydrolyzed in the process. Rennin is completely precipitated from its solution by saturating the liquid with sodium chloride. All these properties are characteristic of acid albumins.

Summary

Pepsin and rennin are proteins possessing widely different properties. Pepsin is coagulated by heat and is colloidal in nature, while rennin is a decomposition product of protein of the acid albumin type, and is not

precipitated on boiling. The former may be dialyzed, while the latter diffuses readily through parchment membranes. Proteolytic or peptic activity does not seem to be a part of the true physiological characteristics of the milk-curdling enzyme. These conclusions are based on the facts that pepsin digests 25,000 times its own weight of freshly coagulated and disintegrated egg albumen in $2^1/_2$ hours at 52°, the assay method of the U. S. Pharmacopeia, and that rennin is capable of coagulating more than 2,000,000 times its weight of fresh milk in 10 minutes at 40°.

Both enzymes are present in the stomach of the suckling calf, while in that of the adult hog only pepsin was found.

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NOTE

A Method for the Simultaneous Determination of Sulfur and Halogen in Organic Compounds.—When, in 1886, Peter Klason described a simplification[1] of his then new method[2] for the determination of sulfur in organic compounds, he stated that it probably would not supercede the Carius method, but might serve as a supplement to the latter. Through years of usage, however, in the laboratories of Sweden the process has received further simplification until to-day it surpasses the Carius method and rivals that of fusion in ease, speed and accuracy.

American chemists seem to be totally unacquainted with either the old or the new technique, so a summary of the present modified method as used in the laboratory of Bror Holmberg[3] at the Tekniska Hogskola, Stockholm (and also at the laboratories of Upsala and Lund) may serve to call attention to this facile analysis, useful not only for sulfur alone but for simultaneous determination of sulfur and halogen.

Procedure.—The process is carried out in a glass combustion tube in the combustion furnace. One end of the tube is drawn out and bent down to dip below the surface of 100 cc. of distilled water in a receiver flask. At the opposite end there is attached by a fume-treated cork a bulb of sulfur-free, fuming nitric acid. A stream of dry air or oxygen may be passed through acid and tube.

The latter contains, over the end of the furnace nearest the bent portion of the tube, a roll of freshly-ignited, fine-meshed platinum gauze (A) and then a porcelain boat (B) filled with fuming nitric acid. At the midpoint

[1] Klason, *Ber.*, **19**, 1910 (1886).

[2] Claësson, *Z. anal. Chem.*, **22**, 177 (1883).

[3] This method is used in the following work: Holmberg, Stereokemiska Studier, etc., *Z. anorg. Chem.*, **56**, 385 (1907); *J. Prakt. Chem.*, [2] **71**, 264 (1905); **75**, 170 (1907); **79**, 253 (1909); **81**, 451 (1910); **84**, 634 (1911); **87**, 456 (1913); **88**, 553 (1913); *Ber.*, **47**, 159, 167 (1914); *Ark. Kemi, Min. Geol.*, **6**, Nos. 1, 8, 17, 23 (1915–17); **8**, Nos. 2, 8 (1920–21). Leonard, Diethylrhodanine, *Medd. Vetenskapsakad. Nobelinst.*, **14**, No. 4 (1921).