[CONTRIBUTION FROM THE RESEARCH LABORATORY AND THE DEPARTMENT OF GLANDULAR EXTRACTS, PARKE, DAVIS & CO.]

STUDIES ON PEPSIN. I. CHEMICAL CHANGES IN THE PURIFICATION OF PEPSIN.[1]

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The question of the chemical composition of pepsin has occupied the attention of a number of investigators. Following the classical researches of Pawlow[2] and his pupils, Pekelharing[3] appears to have been the first to undertake purification of the enzyme. This investigator prepared a light yellow powder which, while readily soluble in dilute acids and sodium chloride solution, dissolved with difficulty in water but showed strong peptic activity. It gave reactions for albumin, but was believed to contain a soluble phosphorus compound as an impurity. On boiling pepsin solutions, Pekelharing obtained a nucleoproteid and was able, under certain conditions, to separate an albumose.

Nencki and Sieber,[4] using as initial material juice obtained through gastric fistula in dogs, claim to have secured an active pepsin preparation through precipitation which is free from albumin. At the same time, they consider the precipitate of transparent granules containing chlorine which they obtained by strongly cooling the gastric juice to be a chemical individual, and, in all probability, the true enzyme. They also submit analyses to support their contentions. Pekelharing,[5] in a later investigation, in which he employed the artificial gastric juices extracted from several hundred hog stomachs by his previous method, and also the juice obtained from gastric fistula in dogs, disproved this view. He found pepsin to be free from phosphorus and to contain no nucleoproteid, but the analyses of his preparations showed no constancy in results.

That a protein-free pepsin solution having digestive action is possible, has also been maintained by Schrumpf.[6] The latter prepared a Büchner-pressed extract of hog stomachs, clarified by filtration, and dialyzed against running water. The dialysate thus obtained was precipitated by addition of cholesterin in alcohol-ether solution, filtered, the precipitate redissolved in water, and the suspension finally clarified by a Kitasato candle. The clear filtrate, while giving none of the protein reactions, still showed powerful digestive activity.

[1] Read before the Biological Section of the American Chemical Society at the Cleveland meeting, September 12, 1918.

[2] Pawlow, *Centr. Physiol.*, 1888; *Ergebnisse Physiol.*, **1902**, i, Part I, 246.

[3] Pekelharing, *Z. physiol. Chem.*, **22**, 233 (1897).

[4] Nencki and Sieber, *Ibid.*, **23**, 291 (1901).

[5] Pekelharing, *Ibid.*, **35**, 8 (1902).

[6] Schrumpf, *Beitr. Hofm.*, **6**, 396 (1905).

The amino acid constituents of pepsin have been investigated by Hugounenq and Morel[1] using an autodigested, hydrochloric extract of hog stomachs. They conclude that an extract of pepsin contains a number of monoamino acids in the free state, probably formed in the autodigestion. Glycocoll, aspartic and glutaminic acids, and also histidin, they found to be absent in the material examined.

It is thus readily apparent that, as true with other enzymes, the chemical nature of pepsin is still an open question. Nearly all of the above investigators have based their conclusions on crude preparations, undoubtedly containing admixed or combined impurities. Seemingly, no attempt has been made to prove, by quantitative measurements of the proteolytic activity, that an actual purification has taken place, where such is mentioned. The present investigation was undertaken by us to determine what changes take place in the purification of pepsin, with the view of possibly throwing some light on the chemical nature of the enzyme.

Experimental Procedure.

Methods.—As basic material for purification, a composite lot (consisting of a number of different samples) of 1 : 2000 commercial pepsin was employed. Sufficient stock of this mixture was reserved to enable the preparation of all of the various strengths of the enzyme given below. The weaker samples (up to 1 : 18000) were prepared by fractional precipitation of a 20% aqueous solution, while the more active strengths were obtained by salting out the former, filtering and dialyzing. In each case, the final purified material was dried to a constant moisture content of about 5% and scaled. Assays for proteolytic power were then carried through and the samples analyzed chemically.

Determination of the proteolytic strength of the different samples, made in association with our colleagues, L. M. Gerdes and W. L. Seibert, was in accordance with the method given in the 9th revision of the U. S. Pharmacopoeia.[2] The assays were checked in each case, and controlled by running through a standard (1 : 3000) pepsin under identical conditions.

The chemical examination included analyses of total mineral matter, total nitrogen, total sulfur by the method of Wolf and Osterberg,[3] volumetric estimation, in the ash, of phosphoric acid as P_2O_5,[4] chlorides as NaCl,[5] calcium as CaO; also, determination of nitrogen existing in coagulable protein, proteoses by zinc sulfate precipitation,[6] peptones by Bigelow

[1] Hugounenq and Morel, *Compt. rend.*, **147**, 212 (1908).

[2] "Pharmacopoeia of the United States," **1916**, p. 312, 9th Rev., P. Blakiston's Son & Co.

[3] Wolf and Osterberg, *Biochem. Z.*, **29**, 429 (1910).

[4] "Methods of Analysis, A. O. A. C.," U. S. Dept. Agr., Bur. Chem., *Rev. Bull.* 107, 4 (1912).

[5] "Standard Methods of Water Analysis," *Am. Pub. Health Ass'n*, **1917**, p. 4.

[6] Bömer, *Z. anal. Chem.*, **5**, 562 (1895).

TABLE I.—ANALYSES.

Proteolytic. strength (U. S. P. IX.)	Total mineral matter. %	Phosphoric acid as P_2O_5. %	Calcium as CaO. %	Chlorides as NaCl. %	Total sulfur. %	Percentage of nitrogen in					Optical rotation α_D at 24°.	Reaction C_{H^+} at 23°.
						Total. %	Coagulable protein. %	Proteoses. %	Peptones. %	Amino acids. %		
1:2000	5.37	1.58	0.26	1.19	0.63	12.93	1.15	0.73	7.37	4.39	—2° 58′	.....
1:5500	4.31	1.42	0.32	Trace	0.70	12.60	1.41	1.76	4.78	4.04	—2° 0′	.....
1:6000	3.34	1.03	0.46	Trace	0.81	13.41	1.43	2.10	4.91	3.15	—2° 6′	.....
1:10,000	3.31	1.42	0.35	Trace	0.89	13.55	1.63	3.00	3.73	3.09	—2° 24′	.....
1:12,000	2.31	1.28	0.29	Trace	0.63	12.95	2.33	3.09	3.41	2.75	—2° 10′	.....
1:18,000	2.84	1.47	0.71	Trace	1.50	13.47	3.16	3.62	2.68	2.10	—2° 30′	.....
1:21,000	2.38	1.29	0.58	Trace	0.82	12.57	3.69	3.91	3.73	1.45	—2° 0′	.....
1:24,000	2.84	1.27	0.52	Trace	0.77	12.64	3.98	4.10	0.96	1.35	—2° 4′	2.5×10^{-4}
1:28,000	1.86	1.09	0.53	None	1.62	13.72	4.39	4.32	0.78	1.22	—2° 20′	4.0×10^{-5}
1:40,000	2.01	0.47	1.01	None	1.50	13.77	8.34	4.43	..	0.61	—2° 30′	6.0×10^{-7}

and Cook's[1] modification of Sjerning's method, and amino acids according to Van Slyke.[2] In addition, observations were made in a 2% aqueous solution of optical rotation, and of the hydrogen-ion concentration. The direct reading ionometer described by Bartell[3] was used in the latter, with a Weston Standard Cell, and the chain: Calomel electrode (*N* KCl)—saturated KCl—pepsin solution—Pt electrode—H_2 at 23°. The complete "set up" employed was similar to that used by Davis[4] in a previous investigation of diphtheria toxin.

Supplementing the preceding, qualitative tests were carried out in accordance with the technique employed by one of us, Davis,[5] with peptone samples. Both a straight 2% aqueous solution and the filtrate, after coagulating the protein, were used, and examination made for: tyrosin (xanthoproteic, Millon's reaction) tryptophane (Adamkiewicz Hopkins-Cole reagent), glycoprotein and glycoproteose (Molisch reagent). Tests were also made on the filtrate from coagulable protein, for proteoses (by addition of saturated zinc sulfate, ammonium sulfate, picric acid solutions), and protoproteoses (by saturated sodium chloride solution, potassium ferrocyanide in acetic acid solution).

Results.—Altogether, nine purified products were prepared. Including the basic pepsin material, the various proteolytic strengths of the enzyme which were examined ranged from 1 : 2000 to 1 : 40,000 (U. S. P. IX). The results given in the accompanying Tables I and II, are, in every case, based on duplicate determinations and, because of possible variation in the U. S. P. pepsin assay, these estimations were carried out in triplicate by two different observers.

As may be noted from Table I, the purification of pepsin is accompanied by a general decrease in the total mineral matter. This ranges from an ash content of nearly 5.5% in the case of the basic (1 : 2000) product down to about 2% with the highest proteolytic strengths obtained. The phosphoric acid content, also, shows a gradual decrease so that the value at 1 : 40,000 is less than one-third that of the basic material. Both the calcium oxide and total sulfur values fluctuate in the different strengths but both show an increase in the purified as compared with the unpurified samples. It is a significant fact that the chlorides, which are present to the extent of 1.19% (as NaCl) in the 1:2000 sample, practically disappear as a result of purification.

Probably the most important data are furnished by the various nitrogen factors, particularly the nitrogen in amino acid condition. Confirming

[1] Bigelow and Cook, THIS JOURNAL, **38**, 1496 (1906).
[2] Van Slyke, *J. Biol. Chem.*, **16**, 121 (1913).
[3] Bartell, THIS JOURNAL, **39**, 630 (1917).
[4] Davis, *J. Lab. Clin. Med.*, **3**, 358 (1918).
[5] Davis, *Ibid.*, **3**, 75 (1917).

TABLE II.—REACTIONS IN 2% SOLUTION.

Proteolytic strength. (U. S. P. IX.)	Character of solution.	Picric acid reaction.	Ammonium sulfate reaction.	Millon's reagent.	Biuret reaction.	Hopkins-Cole reagent.	Molisch's reagent.	Xanthoproteic reaction.
1:2,000	Straight			Mod. ppt., reddish	Bluish pink	Ppt., ring	Ppt., ring	Yellow color ppt., orange
	Coag. filtrate	Mod. ppt.	No ppt.	Sl. ppt., reddish	Bluish pink	No ppt., ring	Ppt., ring	Yellow, deep orange
1:5,500	Straight			Heavy ppt., pink	Bluish pink	Ppt., ring	Ppt., ring	Ppt., orange
	Coag. filtrate	Mod. ppt.	No ppt.	Sl. ppt., reddish	Bluish pink	No ppt., ring	Ppt., ring	No ppt., mod. orange
1:6,000	Straight			Mod. ppt., pink	Bluish pink	Ppt., ring	Ppt., ring	Ppt., mod. orange
	Coag. filtrate	Mod. ppt.	No ppt.	Sl. ppt., pink	Bluish lavender	No ppt., ring	Ppt., ring	No ppt., deep orange
1:10,000	Straight			Heavy ppt., pink	Bluish pink	Ppt., ring	Ppt., ring	Ppt., deep orange
	Coag. filtrate	Mod. ppt.	No ppt.	Sl. ppt., pink	Bluish lavender	No ppt., ring	Ppt., ring	No ppt., light orange
1:12,000	Straight			Heavy ppt., pink	Bluish pink	Ppt., ring	Ppt., ring	Ppt., orange
	Coag. filtrate	Mod. ppt.	No ppt.	Sl. Ppt., pink	Bluish lavender	No ppt., ring	Ppt., ring	No ppt., light orange
1:18,000	Straight			Heavy ppt., reddish	Bluish pink	Ppt., ring	Ppt., ring	Ppt., orange
	Coag. filtrate	Mod. ppt.	No ppt.	Sl. ppt., pink	Bluish lavender	No ppt., ring	Ppt., ring	No ppt., red-orange
1:21,000	Straight			Heavy ppt., pink	Bluish pink	Ppt., ring	Ppt., ring	Ppt., red-orange
	Coag. filtrate	Mod. ppt.	Sl. opal	Sl. ppt., red tinge	Bluish lavender	No ppt., ring	Ppt., ring	No ppt., orange
1:24,000	Straight			Heavy ppt., pink	Bluish pink	Ppt., ring	Ppt., ring	Ppt., red-orange
	Coag. filtrate	Sl. ppt.	Sl. opal	Sl. ppt., red tinge	Bluish lavender	No ppt., ring	Ppt., ring	No ppt., yellow-orange
1:28,000	Straight			Heavy ppt., pink	Bluish lavender	Ppt., ring	Ppt., ring	Ppt., red-orange
	Coag. filtrate	Sl. ppt.	Sl. opal	Trace ppt., red tinge	Bluish lavender	No ppt., trace ring	Ppt., ring	No ppt., faint reddish
1:40,000	Straight			Heavy ppt., pink	Bluish ppt., lavender	Ppt., ring	Ppt., ring	Ppt., red-orange
	Coag. filtrate	Opal	Opal	Opal red tinge	Bluish, no color	No ppt., no ring	Ppt., ring	No ppt., faint reddish

more elaborately the results found by Aldrich,[1] there is found to be almost a uniform decrease in α-amino acid nitrogen so that in the sample testing 1:40,000 only 0.61% is found. Corroborating these results, it will be noted from the table that there are steady increases in both the coagulable protein nitrogen and that existing as proteoses, while the peptone nitrogen like that of the amino acids shows a decrease. The values for total nitrogen showed decided variations among the different samples with no significant change as the purification increases.

All of the different strengths of the pepsin examined show levorotation in very nearly the same degree so that this factor is apparently unaltered as a result of purification. With the exception of the strongest sample obtained (1 : 40,000) a slight amount of hydrochloric acid was used in the preparation of the other strengths of the pepsin. As a consequence, 2% aqueous solutions of these samples show relatively high hydrogen-ion concentration. However, the reaction of the 1 : 40,000 sample, which is the nearest approach to the pure enzyme, is very nearly neutral ($C_H{}^+ = 6.0 \times 10^{-7}$). This would tend to disprove the view held by Jacoby[2] and others that pepsin is an acid.

Consideration of the data presented in Table II shows that the results corroborate, in a general way, the analytical data already discussed. No tests were made on the straight pepsin solutions with saturated picric acid, sodium chloride and ammonium sulfate solutions, and also none with potassium ferrocyanide in acetic acid solution, since the results with all of these reagents, because of coagulable protein would be positive, and practically the same for the different strengths. Confirming the results given in Table I, the saturated picric acid, Hopkins-Cole, and Millon's reagent tests, made of the filtrate after removal of coagulable protein, show presence of amino acid and peptid bodies in the lower strength samples. These gradually disappear so that only traces are found in the highest strength sample of the enzyme. Both saturated sodium chloride solution and potassium ferrocyanide in acetic acid solution gave negative results, indicating absence of protoproteoses in the filtrate from coagulable protein. A positive reaction was obtained in every case with Molisch reagent showing presence of glycoprotein, or its derivatives, in the material. It is significant that the biuret test of the filtrate after coagulation of protein in the 1 : 40,000 sample, is negative. This would indicate that practically all of the protein material is of the nature of coagulable protein or even more complex in its protein character.

Discussion.

A review of the data presented in the foregoing seems to show that in the purification of pepsin there is a gradual elimination of the secondary

[1] Aldrich, *J. Biol. Chem.*, **23**, 339 (1915).

[2] Jacoby, *Biochem. Z.*, **4**, 471 (1907).

protein derivatives including amino acids. This is manifested by a constant tendency in the purified samples to approach nearer to the actual character of proteins with increasing proteolytic activity, and is accompanied by an increase in material coagulable by heat. From the fact that the highest strength samples still give strong tests with Molisch reagent, it may be possible that the pure enzyme is a conjugated protein, probably a glycoprotein.

Confirming this view, the mineral matter is decidedly less in the purified samples than in the original basic material, approaching almost to the value for pure proteins in the case of the strongest samples. Both sulfur and calcium are probably unaffected by the purification, but there is a decided decrease in the phosphorus content and seemingly a total elimination of chlorides. Other than the increase which would obtain by removal of non-nitrogenous impurities, there is probably not much change in the content of total nitrogen as a result of pepsin purification.

The manner in which the α-amino acids decrease as the proteolytic activity increases is striking, and seems to be almost proportional in amount. It is noteworthy that the small amount of α-amino acid present in the sample testing 1 : 40,000 (0.61%) very nearly approaches the value for this factor due to lysin as found present by Van Slyke and Birchard[1] in most proteins analyzed by the nitrous acid method.

Results of optical activity determinations are apparently of no significance, since the same values are obtained with several different strengths of pepsin. As already mentioned above, the reaction in aqueous solution of the strongest (1 : 40,000) pepsin is significant because of its very slight acidity. It would seem very likely, that the concentration of hydrogen ions in solutions of the pure enzyme, when isolated, will probably show only the slight acidity comparable to that given by other proteins.

In connection with the assays of proteolytic strength by the U. S. P. method, it was deemed of interest to make a comparison of the rennetic power of the different samples. It is a significant fact that throughout the entire series, from 1 : 2000 to 1 : 40,000, the rennetic activity and proteolytic strengths are found to go hand in hand. This is being investigated, and will be reported upon in a later paper.

Conclusions.

1. The purification of pepsin seems to consist in the elimination of secondary protein derivatives including α-amino acids.

2. Calcium and sulfur appear to be unaltered as a result of purification, but phosphorus is materially reduced. Chlorides are seemingly entirely removed.

3. Aqueous solutions of pepsin, after purification, show no material

[1] Van Slyke and Birchard, *J. Biol. Chem.*, 16, 539 (1914).

change in optical activity. A sample of high digestive power (1 : 40,000), shows a reaction very nearly neutral.

4. Pepsin tends to approach nearer to the actual character of a protein (possibly a glycoprotein) with increasing proteolytic activity.

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THE EFFECT OF NEUTRAL SALTS UPON THE ACTIVITY OF PTYALIN.

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The original purpose of the investigation was to determine the effect of small amounts of ammonium salts upon the activity of ptyalin, but it was later extended to include a comparison with the influence of other neutral salts. It is a continuation of work previously reported[1] on auxoamylases, or activators of amylolytic enzymes. Because of interruptions which will delay the work for some time, it seems best to publish these results now.

The methods used were essentially as described in the previous article (*loc. cit.*). Digestion of portions of a boiled starch solution was carried on at 38° under the same conditions in the presence of toluene. At intervals, 25 cc. were pipetted off, heated with an excess of Fehling's solution, the precipitated oxide filtered out and, after dissolving, its amount determined by addition of an excess of potassium iodide and titration of the freed iodine by decinormal sodium thiosulfate.

The salts used were either the chemically pure, neutral compounds as purchased or were made by neutralizing known amounts of the corresponding acids with ammonium hydroxide. Neutrality of the digesting mixture was maintained by the use of a dialyzed litmus solution. Determinations were made in duplicate and only the averages of closely agreeing ones are given. Except in the same series different specimens of saliva were employed and therefore the results of different series are not strictly comparable.

The figures given are relative numbers. They represent the number of cubic centimeters of the thiosulfate solution required for 25 cc. of the digesting mixture. Inasmuch as this corresponds to the amount of cuprous oxide, and the latter to the hydrolytic products from the starch, the figures stand for the digestive activity of the enzyme under different conditions. The standard used for comparison contained water instead of one of the salt solutions, but was otherwise the same.

The solutions were all made up by adding to 180 cc. of a 1% starch solution 10 cc. of a 2 or 3% solution of saliva and 10 cc. of a salt solution of

[1] THIS JOURNAL, 39, 2745 (1917).