THE PURIFICATION OF PEP SIN, ITS PROPERTIES, AND
PHYSICAL CHARACTERS.

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(Received for publication, October 8, 1926.)

Since 1836, when Theodor Schwann signalized the presence in gastric
juice of the proteolytic ferment which he was the first to designate as pep-
sin, a large number of attempts have been made to isolate it in a pure form
to determine its chemical nature and its ultimate physiological properties.
In a large majority of these, and, especially, all those made before 1895,
except that of Brücke (1), who was the first to use adsorption, the methods
used would not now be regarded as adequate for the purpose, and the results
they gave are, accordingly, now only of historic interest in relation to the
subject. In consequence a detailed reference to them will be omitted here
and only such citations of the later publications to which we owe what at
present we know about pepsin will be made in this contribution on the
subject.¹

Pekelharing (2), by dialyzing, at low temperature, against distilled
water, gastric juice from a dog with a Pavlov fistula, till the concentration
of hydrochloric acid in the juice was decreased to about 0.02 per cent, pre-
cipitated the pepsin therein as a finely divided suspension. This suspen-
sion he separated by centrifuging and, after washing the sediment with
distilled water, dried it in a desiccator. From the clear fluid left after
centrifuging he precipitated, by half saturation with ammonium sulfate,
more pepsin which was then freed from this salt by dialysis and, after the
addition of hydrochloric acid to the extent of 0.2 per cent, was subjected
to dialysis against distilled water, thus furnishing an additional quantity
of pure pepsin. The average composition of the pepsin so prepared was
C 51.99, H 7.07, N 14.44, S 1.63, Cl 0.49, P 0.01 per cent.

The phosphorus found in pepsin Pekelharing regarded as due to traces of
an impurity resulting from the digestion of a phosphorus-containing pro-
tein. The chlorine, on the other hand, he found to be constant, even in
preparations obtained by dialysis with oxalic acid.

He obtained a coagulated product of an acid character on heating either
gastric juice or purified pepsin in acid solution, to which he gave the name

¹ An extended account of the literature of the subject is given in Oppen-

THE JOURNAL OF BIOLOGICAL CHEMISTRY, VOL. LXXI, NO. 3
Purification of Pepsin

peptic acid. It was soluble in alkaline solutions but insoluble in dilute acids. It was soluble in water and in hot alcohol. It gave the biuret, Millon's, and Adamkiewicz's reactions but, when in solution, did not give off its sulfur on heating with alkali. Its chemical composition was C 50.79, H 7.02, N 14.44, S 1.08 per cent.

Nencki and Sieber (3), by dialyzing at a low temperature fresh gastric juice from a gastric fistula made by Pavlov's method, obtained a precipitate which contained chlorine, iron, and phosphorus, and which on heating with hydrochloric acid yielded a nucleoprotein and an albumose. The nucleoprotein on hydrolysis with sulfuric acid yielded purine bases and pentoses. On washing the precipitate with alcohol, the chlorine was decreased and a lecithin-like substance was extracted. It was found that the content of iron and phosphorus was inconstant, but, nevertheless, they believed they had isolated pepsin in a state of purity, that the inconstancy of its composition was due to its labile character, and that its molecule consists of a nucleoprotein, lecithin, phosphoric acid, chlorine, and iron. The ultimate analysis of their pure product gave the following values: C 51.26, H 6.74, N 14.33, Cl 0.476, P 0.104 per cent.

The ultimate composition of pepsin, as determined by Pekelharing and Nencki and Sieber on their preparations of it, would appear to indicate that it is a protein. Ringer (4), however, holds that in the purest preparation of pepsin which he made by dialysis there was a mixture of protein and the enzyme proper as he found that in the electric field the former migrated to the cathode while the pure product, which was very actively digestive, passed always to the anode and had no isoelectric point. He did not prepare any of this product for analysis, and its composition was, therefore, undetermined, but he found his purest preparations free from chlorine and iron. Hammarsten (5), by the use of methods which gave him preparations of pepsin as active as those obtained by Pekelharing, but of which he made no elementary analyses, generalized from his observations on these that pure pepsin is always an enzyme-protein compound. Davis and Merker (6), by salting out aqueous solutions of commercial pepsin, filtering, and dialyzing, obtained very active preparations of pepsin (1:40,000 as compared with 1:3000 U.S.P. standard) which contained 2.01 per cent mineral matter, no chlorine, 0.47 per cent P₂O₅, 1.01 per cent CaO, and 1.5 per cent S. The nitrogen content was 13.77 per cent and that in α-amino acid form was only 0.61 per cent. Of the protein reactions the Molisch was marked and this, with the other characters manifested by the product, led Davis and Merker to regard pepsin as a glycoprotein.

Effront (7), by using paper pulp made from certain types of Dreverhoff's filters (No. 311) as an adsorbent, obtained very active preparations of pepsin which contained only 0.4 per cent nitrogen and with an inorganic (ash) content of 1.6 per cent. He reported no analyses of the product.

From this all too brief review of the literature of the subject it will appear that the chemical and other characters of pepsin are as yet not definitely determined and that further research is
necessary in order to establish what pepsin is. It was with the hope of adding somewhat to our knowledge of its chemical constitution and physical properties that the author undertook an investigation on it, and he now presents for record the results which he has obtained.

Methods of Investigation and the Results.

The investigation was directed to deal with the following:

1. Method of standardization of the digestive power of pepsin.
2. The purification of pepsin involving: (a) The determination of a suitable adsorbent; (b) adsorption with aluminium hydroxide and subsequent liberation and precipitation; (c) precipitation with safranine and removal of the safranine with isoamyl alcohol.
3. Chemical analyses of the various preparations.
4. (a) Hydrolysis of purified pepsin and determination of the various amino acids and purine bases resulting as well as the nitrogen distribution therein; (b) color reactions given by the various preparations.
5. Determination of the isoelectric point.
6. Influence of pepsin on the conductivity and hydrogen ion concentration of acid solutions of the same.
7. Effect of extraction with ether on the activity of pepsin.
8. Effect of alcohol-ether mixtures on solutions of pepsin at various hydrogen ion concentrations.

1. Method Employed in Standardizing Peptic Activity.

The method used in determining the peptic activity of the various preparations is that commonly known as the Mett tube method. A series of glass tubes, from 1 to 2 mm. in diameter, was filled with fresh white of egg and the albumen coagulated at about 90°C. The tubes were cut as required into fragments 10 to 15 mm. in length, and two of these placed in the enzyme liquid to be examined. The preparations were then placed in an incubator at about 37°C. for 24 hours, after which the tubes were removed and the length of albumen dissolved at both ends carefully measured. The temperature of the incubator varied considerably from one determination to another. However,
since each complete series was carried on simultaneously, all the results of such a series can be compared, but on account of the fluctuations of the temperature from time to time, the results of different series are not necessarily comparable.

Schutz (8) and Borissow (9) investigated this method and they found that within certain limits the rate of digestion, or the lengths of the column of albumen dissolved in the same time interval, is proportional to the square root of the quantity of enzyme. In other words, if \( M \) and \( M' \) are the lengths of albumen dissolved in the same time interval, the ratio of their concentrations is \( 1 : \left( \frac{M'}{M} \right)^2 \).

2. Purification of Material.

(a) Preliminary experiments were carried out on the adsorption of pepsin by various organic and inorganic adsorbents in the hope that one would prove satisfactory. The various adsorbents used were animal charcoal, kaolin, Lloyd's reagent, cholesterol, camphor, salicylic acid, acetonilide, aluminium oxide, and freshly precipitated aluminium hydroxide. The results obtained from these determinations showed that cholesterol, camphor, salicylic acid, acetonilide, and aluminium oxide adsorb only traces of the active principle; kaolin and animal charcoal adsorb it partially from both neutral and acid solutions; Lloyd's reagent adsorbs it slightly from neutral solutions but very markedly from acid solutions. The pepsin adsorbed by Lloyd's reagent could be liberated only with great difficulty, consequently this reagent was not applicable for the preparation of the enzyme in a pure form. Freshly precipitated aluminium hydroxide, on the other hand, was found to be not only a very efficient adsorbent but the adsorbed pepsin could be readily liberated by dissolving the aluminium hydroxide in dilute hydrochloric acid. This adsorbent was therefore used in one of the methods of purification employed. The results obtained with it are given below.

Adsorption with Freshly Precipitated Aluminium Hydroxide.

Experiment 1.—2 gm. of aluminium chloride were dissolved in 100 cc. of distilled water and the aluminium precipitated, in the cold, with concentrated ammonium hydroxide. The precipitated
aluminium hydroxide was centrifuged off and washed several times with distilled water. It was then added to a solution of pepsin in distilled water (10 gm. of pepsin in 150 cc. of water). The solution was first distinctly acid to litmus, but on standing for a few hours in contact with the aluminium hydroxide the hydrogen ion concentration of the solution decreased to pH 4.5 ± 0.3. The precipitate was then centrifuged off and the activity of the supernatant liquid compared with that of the original solution. 1 cc. of each solution was diluted with 7.5 cc. of 0.2 per cent hydrochloric acid. The digestive power of these diluted solutions was then determined with Mett tubes.

<table>
<thead>
<tr>
<th>Digestion</th>
<th>Ratio of concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original solution</td>
<td>9.0</td>
</tr>
<tr>
<td>Supernatant liquid</td>
<td>2.5</td>
</tr>
<tr>
<td>Amount adsorbed</td>
<td>92.2 per cent.</td>
</tr>
</tbody>
</table>

Experiment 2.—1 gm. of aluminium chloride was dissolved in 75 cc. of water. The aluminium was precipitated with ammonium hydroxide and washed as described in Experiment 1. The aluminium hydroxide was then added to a solution of pepsin in water (10 gm. of pepsin in 150 cc. of water) and allowed to stand, with occasional shaking, for several hours. The aluminium hydroxide was then centrifuged off and the activity of the supernatant liquid compared with that of the original solution.

<table>
<thead>
<tr>
<th>Digestion</th>
<th>Ratio of concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original solution</td>
<td>8.0</td>
</tr>
<tr>
<td>Supernatant liquid</td>
<td>4.0</td>
</tr>
<tr>
<td>Amount adsorbed</td>
<td>75 per cent.</td>
</tr>
</tbody>
</table>

Experiment 3.—5 gm. of pepsin were dissolved in 75 cc. of N/10 HCl and about 5 gm. of freshly prepared and washed aluminium hydroxide added. The acidity of the solution fell in a couple of hours to pH 4.5 ± 0.3 on account of the basic action of the aluminium hydroxide. After centrifuging off the aluminium hydroxide, the digestive action of the supernatant liquid was compared with that of the original solution.

<table>
<thead>
<tr>
<th>Digestion</th>
<th>Ratio of concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original solution</td>
<td>10</td>
</tr>
<tr>
<td>Supernatant liquid</td>
<td>1.25</td>
</tr>
<tr>
<td>Amount adsorbed</td>
<td>98.5 per cent.</td>
</tr>
</tbody>
</table>
(b) Preparation by Adsorption with Aluminium Hydroxide.—
Artificial gastric juice was prepared in the usual manner by digesting finely minced stomach mucosa of pigs, for several days, in 0.4 per cent hydrochloric acid and then filtering through a thick layer of paper pulp. The filtrate though yellow was perfectly clear. About 20 gm. of freshly precipitated and washed aluminium hydroxide were then added per 4 liters of solution. After a few hours, with occasional shaking, the supernatant liquid was decanted and the aluminium hydroxide separated by centrifuging. It was then placed in collodion dialyzing tubes and dissolved by dialyzing against 0.5 per cent hydrochloric acid for several days. A fairly large residue of organic material remained undissolved and was filtered off. The filtrate was dialyzed against tapwater until a small precipitate formed. This precipitate was centrifuged off and the dialysis continued against tap water until a fairly large precipitate separated out. This precipitation reached a maximum at about pH 3.5. The digestive power of the precipitates was determined in various samples prepared and compared with that of standard pepsin 1:3000 U.S.P. The first precipitate never exhibited more than a very slight digestive action, but the second showed activities varying from 1:40,000 to 1:42,000 U.S.P. On several occasions this precipitate was snow-white but it had generally a slightly yellow color. This preparation was analyzed and will be referred to as Preparation I.

Pepsin prepared in the manner described above was further purified as follows: It was dissolved in 0.2 per cent hydrochloric acid and dialyzed against a large volume of distilled water. A small portion of the active principle was thus precipitated, but the main portion remained in solution. After centrifuging off the precipitate, the remainder of the active principle was precipitated by adding a small quantity of a neutral lead acetate solution. The precipitate was centrifuged off after a few hours and dissolved in a small volume of a saturated oxalic acid solution. The lead oxalate precipitate which resulted was filtered off and the filtrate dialyzed against distilled water. The dialyzing solution was

2 Tap water was used for the dialysis as it was found to give better results than distilled water. Although the solution became very opalescent when distilled water was used as the dialyzing medium only a very small amount of the active principle could be removed by centrifuging.
changed several times and the dialysis continued until no traces of oxalates could be detected in the pepsin solution. A small precipitate generally formed during the dialysis; this was transferred with the solution to a large watch-glass, evaporated at room temperature, and finally dried in a desiccator over sulfuric acid. Preparations made in this manner varied from 1:39,000 to 1:45,000 U.S.P. This preparation will be referred to as Preparation II.

Although this method of purification decreased the percentage of ash and gave a very active product, it cannot however be considered as wholly satisfactory. There was always a great loss of the active principle during the dialysis. In some cases this loss reached to about 75 per cent of the original material. In the final stages of the dialysis, when the hydrogen ion concentration of the solution was low, the dialyzing liquid generally became opalescent although it manifested only very slight digestive action. It is possible that the enzyme, even at the hydrogen ion concentration of distilled water, may be broken down into fractions which would pass through collodion membranes. The removal of the fractions by dialysis would enable the decomposition to proceed to a great degree, and this would explain the great loss in material during the treatment, but with no corresponding increase in the digestive power of the preparation. Losses of like magnitude were obtained by simply dissolving the pepsin in 0.2 per cent hydrochloric acid, dialyzing against distilled water for several days, and evaporating the solution to dryness at room temperature.

(c) Precipitation with Safranine.—Marston (10), in 1923, showed that all the proteolytic enzymes were quantitatively precipitated from water solutions by safranine and indulin, water-soluble dyes. He made quantitative determinations on the proteolytic activity of his trypsin dye preparation, but he does not appear to have determined the activity of his pepsin preparations. He made, it would seem, no serious attempt at separating the dye from the enzyme. In view of these results of Marston, it was decided to find out if this method could be employed in the purification of pepsin.

20 gm. of commercial pepsin were dissolved in about 350 cc. of distilled water and about 50 cc. of a 1 per cent solution of safra-
nine added. The pepsin was thus quantitatively precipitated. This precipitate was centrifuged off and washed several times with water by centrifuging. Its proteolytic activity was determined after drying and found to be 1:46,000 U.S.P.

Although this material gave very strong digestive action it was very insoluble in acid. In some cases it required almost 24 hours to dissolve 4 mg. in 10 cc. of 0.2 per cent hydrochloric acid. In the subsequent experiments the freshly precipitated pepsin-dye preparation was washed several times, by centrifuging, with approximately 0.1 per cent dye solution and finally with distilled water. The residue was then transferred, without drying, to a beaker and dissolved in a very weak oxalic acid solution in 20 per cent alcohol.

The dye can be almost completely removed from these solutions by repeated extractions with butyl or isoamyl alcohol. Butyl alcohol, however, is not satisfactory because in concentrated enzyme solutions an insoluble precipitate separates out which probably is altered pepsin. Isoamyl alcohol containing about 10 per cent ether gave quite satisfactory results. When the dye extraction was complete, the isoamyl alcohol which had dissolved in the pepsin solution was removed by repeated shaking with ether containing about 10 per cent ethyl alcohol. The pepsin was then precipitated from the solution by the addition of twice its volume of a mixture of equal volumes of ethyl alcohol and ether. The optimum pH for precipitation appears to be in the neighborhood of 2.5 and corresponds to the determined isoelectric point.

This method of preparation gave quite satisfactory results. From 100 gm. of crude commercial pepsin 3 gm. of purified material were obtained which gave a digestive action of 1:64,000 U.S.P. when compared with 1:3000 commercial pepsin. This corresponds to a recovery of about 65 per cent of the active principle present in the original material. This preparation will be referred to as Preparation III.

3. Chemical Analysis.

Preparation I.—(Aluminium hydroxide method.)

Ash.—0.2031 gm. of pepsin gave 0.0067 gm. of ash; therefore ash = 3.3 per cent.
Nitrogen Determination by Kjeldahl Method.

<table>
<thead>
<tr>
<th>Determination No.</th>
<th>Weight of sample.</th>
<th>Weight of N.</th>
<th>N per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>gm.</td>
<td>gm.</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.0898</td>
<td>0.0114</td>
<td>12.70</td>
</tr>
<tr>
<td>2</td>
<td>0.1028</td>
<td>0.0126</td>
<td>12.26</td>
</tr>
<tr>
<td>3</td>
<td>0.0830</td>
<td>0.0105</td>
<td>12.64</td>
</tr>
<tr>
<td>4</td>
<td>0.1008</td>
<td>0.0126</td>
<td>12.50</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td>12.52</td>
</tr>
<tr>
<td>Percentage when calculated on an ash-free basis...</td>
<td></td>
<td></td>
<td>12.94</td>
</tr>
</tbody>
</table>

Carbon and Hydrogen by Combustion.

<table>
<thead>
<tr>
<th>Determination No.</th>
<th>Weight of sample.</th>
<th>Weight of H₂O.</th>
<th>Weight of CO₂.</th>
<th>H per cent</th>
<th>C per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>gm.</td>
<td>gm.</td>
<td>gm.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.1222</td>
<td>0.0748</td>
<td>0.2040</td>
<td>6.80</td>
<td>45.53</td>
</tr>
<tr>
<td>2</td>
<td>0.1351</td>
<td>0.0841</td>
<td>0.2285</td>
<td>6.92</td>
<td>46.13</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td></td>
<td>6.86</td>
<td>45.83</td>
</tr>
<tr>
<td>Percentage when calculated on an ash-free basis...</td>
<td></td>
<td></td>
<td></td>
<td>7.09</td>
<td>47.39</td>
</tr>
</tbody>
</table>

The analysis of Preparation I may be briefly summarized as follows: C 47.39; H 7.09; N 12.94.

Preparation II.—(Aluminium hydroxide and lead acetate method.) Ash.—0.0880 gm. of pepsin gave 0.0004 gm. of ash; therefore ash = 0.45 per cent.

Nitrogen Determination by Kjeldahl Method.

<table>
<thead>
<tr>
<th>Determination No.</th>
<th>Weight of sample.</th>
<th>Weight of N.</th>
<th>N per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>gm.</td>
<td>gm.</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.0815</td>
<td>0.0111</td>
<td>13.62</td>
</tr>
<tr>
<td>2</td>
<td>0.0920</td>
<td>0.0124</td>
<td>13.45</td>
</tr>
<tr>
<td>3</td>
<td>0.1027</td>
<td>0.0138</td>
<td>13.44</td>
</tr>
<tr>
<td>4</td>
<td>0.0986</td>
<td>0.0131</td>
<td>13.29</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td>13.46</td>
</tr>
</tbody>
</table>
Purification of Pepsin

Carbon and Hydrogen by Combustion.

<table>
<thead>
<tr>
<th>Determination No.</th>
<th>Weight of sample (gm.)</th>
<th>Weight of H₂O (gm.)</th>
<th>Weight of CO₂ (gm.)</th>
<th>H per cent</th>
<th>C per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.1433</td>
<td>0.0852</td>
<td>0.2555</td>
<td>6.61</td>
<td>48.63</td>
</tr>
<tr>
<td>2</td>
<td>0.1286</td>
<td>0.0808</td>
<td>0.2297</td>
<td>6.98</td>
<td>48.72</td>
</tr>
<tr>
<td>3</td>
<td>0.1302</td>
<td>0.0815</td>
<td>Lost.</td>
<td>6.95</td>
<td></td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td></td>
<td></td>
<td></td>
<td><strong>6.84</strong></td>
<td><strong>48.67</strong></td>
</tr>
</tbody>
</table>

Sulfur Precipitated as Barium Sulfate.

<table>
<thead>
<tr>
<th>Determination No.</th>
<th>Weight of sample (gm.)</th>
<th>Weight of BaSO₄ (gm.)</th>
<th>S per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.1194</td>
<td>0.0092</td>
<td>1.06</td>
</tr>
<tr>
<td>2</td>
<td>0.2172</td>
<td>0.0168</td>
<td>1.06</td>
</tr>
<tr>
<td>3</td>
<td>0.2085</td>
<td>0.0152</td>
<td>1.00</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td></td>
<td></td>
<td><strong>1.04</strong></td>
</tr>
</tbody>
</table>

Phosphorus Precipitated as Magnesium Ammonium Phosphate.

<table>
<thead>
<tr>
<th>Determination No.</th>
<th>Weight of sample (gm.)</th>
<th>Weight of Mg₃P₂O₇ (gm.)</th>
<th>Weight of Mg₃P₂O₇ corrected (gm.)</th>
<th>P per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.2172</td>
<td>0.0031</td>
<td>0.0022</td>
<td>0.29</td>
</tr>
<tr>
<td>2</td>
<td>0.2085</td>
<td>0.0028</td>
<td>0.0019</td>
<td>0.25</td>
</tr>
<tr>
<td>Blank.</td>
<td>0.0011</td>
<td>0.0007</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td></td>
<td></td>
<td></td>
<td><strong>0.27</strong></td>
</tr>
</tbody>
</table>

The analysis of Preparation II may be briefly summarized as follows:

<table>
<thead>
<tr>
<th></th>
<th>Ash per cent</th>
<th>N per cent</th>
<th>C per cent</th>
<th>S per cent</th>
<th>P per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ash</td>
<td>0.45</td>
<td>N</td>
<td>48.67</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>6.84</td>
<td>P</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Preparation III.—(Safranine precipitation method.)

<table>
<thead>
<tr>
<th>Determination No.</th>
<th>Weight of sample (gm.)</th>
<th>Weight of ash (gm.)</th>
<th>Ash per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.1402</td>
<td>0.0017</td>
<td>1.21</td>
</tr>
<tr>
<td>2</td>
<td>0.1542</td>
<td>0.0018</td>
<td>1.16</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td></td>
<td></td>
<td><strong>1.19</strong></td>
</tr>
</tbody>
</table>
Iron.—The ash was dissolved in a small volume of concentrated hydrochloric acid, the iron precipitated by ammonia, filtered, ignited, and weighed as Fe₂O₃.

<table>
<thead>
<tr>
<th></th>
<th>Sample 1</th>
<th>Sample 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight of sample</td>
<td>0.1402</td>
<td>0.1542</td>
</tr>
<tr>
<td>&quot; ash</td>
<td>0.0017</td>
<td>0.0018</td>
</tr>
<tr>
<td>&quot; Fe₂O₃</td>
<td>0.0009</td>
<td>0.0009</td>
</tr>
</tbody>
</table>

Average per cent of iron in total sample, 0.43

Nitrogen by Kjeldahl Method.

<table>
<thead>
<tr>
<th>Determination No.</th>
<th>Weight of sample</th>
<th>Weight of N.</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>gm.</td>
<td>gm.</td>
<td>per cent</td>
</tr>
<tr>
<td>1</td>
<td>0.1487</td>
<td>0.01706</td>
<td>11.47</td>
</tr>
<tr>
<td>2</td>
<td>0.0870</td>
<td>0.01008</td>
<td>11.59</td>
</tr>
<tr>
<td>3</td>
<td>0.0918</td>
<td>0.01017</td>
<td>11.08</td>
</tr>
</tbody>
</table>

Average............................................ 11.37

Percentage calculated on an ash-free basis........ 11.50

Carbon and Hydrogen by Combustion.

<table>
<thead>
<tr>
<th>Determination No.</th>
<th>Weight of sample</th>
<th>Weight of H₂O.</th>
<th>Weight of CO₂</th>
<th>H</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>gm.</td>
<td>gm.</td>
<td>gm.</td>
<td>per cent</td>
<td>per cent</td>
</tr>
<tr>
<td>1</td>
<td>0.1007</td>
<td>0.0555</td>
<td>0.1647</td>
<td>6.12</td>
<td>44.60</td>
</tr>
<tr>
<td>2</td>
<td>0.1090</td>
<td>0.0600</td>
<td>0.1790</td>
<td>6.12</td>
<td>44.79</td>
</tr>
</tbody>
</table>

Average............................................ 6.12  44.70

Percentage calculated on an ash-free basis........ 6.19  45.24

Sulfur Precipitated as Barium Sulfate.

<table>
<thead>
<tr>
<th>Determination No.</th>
<th>Weight of sample</th>
<th>Weight of BaSO₄</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>gm.</td>
<td>gm.</td>
<td>per cent</td>
</tr>
<tr>
<td>1</td>
<td>0.0960</td>
<td>0.0128</td>
<td>1.82</td>
</tr>
<tr>
<td>2</td>
<td>0.1260</td>
<td>0.0158</td>
<td>1.72</td>
</tr>
</tbody>
</table>

Average............................................ 1.77

Percentage calculated on an ash-free basis........ 1.79
The analysis of Preparation III, calculated on an ash-free basis, may be briefly summarized as follows:

<table>
<thead>
<tr>
<th></th>
<th>per cent</th>
<th></th>
<th>per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>45.24</td>
<td>N</td>
<td>11.50</td>
</tr>
<tr>
<td>H</td>
<td>6.19</td>
<td>S</td>
<td>1.79</td>
</tr>
</tbody>
</table>

4. (a) Hydrolysis of Purified Pepsin and Determination of Various Amino Acids and Purine Bases Resulting as Well as Nitrogen Distribution Therein.

The Andersen and Roed-Müller (11) modification of Van Slyke's (12) method for determining the nitrogen distribution in proteins was followed for these determinations. This method is given in detail by Plimmer (13) and consequently need not be given here. The results obtained from these determinations are briefly recorded in Table I.

**Hydrolysis and Isolation of Resulting Amino Acids.**—6.5 gm. of pepsin Preparation II were hydrolyzed with sulfuric acid and the

3 This preparation was slightly colored from traces of the dye. Analyses of the safranine used were carried out by the Kjeldahl method, but the nitrogen obtained was only about one-half of the theoretical value. In all probability the nitrogen of the amino groups was alone converted in the digestion. Consequently the nitrogen value obtained for the pepsin may be slightly low. However, it should be noted that in the analysis of the pepsin the carbon remains low; if much safranine was present it would undoubtedly be high, because safranine contains about 73 per cent carbon.
hydrolysate divided into various fractions by Dakin's (14) butyl alcohol method. A number of amino acids was isolated from these various fractions and identified microscopically. The usual method of separation and identification of the diamino and dicarboxylic acid was employed. On account of the small amount of material available it was not found possible to separate definitely the various monoamino-monocarboxylic acids; tyrosine alone was isolated. It was present in fairly large amounts. The various amino acids isolated were: tyrosine, proline, glutamic acid, aspartic acid, histidine, lysine, and possible traces of arginine.

_Determination of Purine Bases._—2 gm. of pepsin were hydrolyzed with dilute sulfuric acid and from the hydrolysate infinitesimal traces of purine bases were isolated by precipitation with ammoniacal silver nitrate. The precipitate gave the nitric acid test for xanthine or guanine, but the Wheeler-Johnson (15) reaction for uracil and cytosine was negative.

(b) Color Reactions Given by Preparations II and III.

Pepsin Preparations II and III were examined for the various protein color reactions, and positive results were obtained with the following tests: Biuret, Millon's, xanthoproteic, Pauly's diazo, Hopkins-Cole, Molisch, and Elliott and Macallum (16). Preparation II, but not Preparation III, reacted positively with the ninhydrin test.

5. Isoelectric Point of Pepsin.

Iscovesco (17), experimenting on the cataphoretic migration of pepsin in artificial and natural gastric juice, obtained results indicating that pepsin in hydrochloric acid concentrations of about 0.4 per cent migrates solely towards the cathode. Michaelis and Davidsohn (18) from similar experiments obtained a hydrogen ion value of $5.5 \times 10^{-2}$ for the isoelectric point of Grübler's commercial pepsin. Pekelharing and Ringer (19), on the other hand, were not able to demonstrate the presence of an isoelectric point when they used pepsin preparations made according to Pekelharing's method. The pepsin migrated towards the anode at all hydrogen ion concentrations of the pepsin solutions.
In view of these conflicting results in regard to the isoelectric point of pepsin, it was considered of interest to determine the isoelectric point of two of the preparations obtained by the author, namely Preparations II and III. These two preparations were made in different ways and one of them had a digestive action approximately 60 per cent greater than the other. A comparison of the digestive powers of these preparations with those obtained by previous investigators indicates that our most active preparation was purer than any which had been previously obtained. This is further supported by a comparison of their chemical analysis.

These two preparations gave practically the same result in regard to the isoelectric point of pepsin. They both manifested anodic migration at pH 3 and above. Although in no case does either show pure cathodic migration, both show a definite preponderance in cathodic migration at a pH of about 2. This would give pepsin an isoelectric point of about 2.5. This value agrees fairly well with the Michaelis and Davidsohn value of pH 2.26 for Grüber's pepsin.

The apparatus used in these determinations was similar to the ordinary cataphoresis apparatus. The pepsin solution was placed in the central chamber and the side chambers filled with the same solution minus the pepsin, the arrangement of the apparatus being briefly thus:

\[
\text{Cu in } \text{CuCl}_2 \text{ solution} \quad \text{Solution} \quad \text{Pepsin solution} \quad \text{Solution} \quad \text{Ag in dilute HCl.}
\]

The voltage was 110.

At the conclusion of an experiment the stop-cocks separating the pepsin solution from that of the side chambers were closed, and a portion of the solution in the side chambers siphoned off. Only the top portion of the solutions was removed, so as to overcome the chance of error from diffusion of the pepsin into the side chambers during the experiment. This was done by filling a small siphon with water with a stop-cock at one end; the other end was then placed about half-way to the bottom of the liquid in the side chamber. On opening the stop-cock the top portion of the liquid was siphoned off. Approximately equal volumes were thus removed from the cathode and anode chambers and
### TABLE II.

**Preparation II. Migration of Pepsin under Influence of an Electric Current.**

<table>
<thead>
<tr>
<th>Determination No.</th>
<th>Normality</th>
<th>Digestion</th>
<th>Ratio</th>
<th>Duration</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
<td>Cathode</td>
<td>Anode</td>
<td>Digestion</td>
</tr>
<tr>
<td>1</td>
<td>0.044</td>
<td>0.048</td>
<td>1.85 mm</td>
<td>2.15 mm</td>
<td>1.0 : 1.2</td>
</tr>
<tr>
<td>2</td>
<td>0.0225</td>
<td>0.0226</td>
<td>2.75 mm</td>
<td>2.75 mm</td>
<td>1.0 : 1.0</td>
</tr>
<tr>
<td>3</td>
<td>0.0225</td>
<td>0.0226</td>
<td>3.25 mm</td>
<td>3.25 mm</td>
<td>1.0 : 1.0</td>
</tr>
<tr>
<td>4</td>
<td>0.0225</td>
<td>0.0226</td>
<td>2.85 mm</td>
<td>3.0 mm</td>
<td>1.0 : 1.1</td>
</tr>
<tr>
<td>5</td>
<td>0.0225</td>
<td>0.0224</td>
<td>1.75 mm</td>
<td>1.50 mm</td>
<td>1.2 : 1.0</td>
</tr>
<tr>
<td>6</td>
<td>0.0136</td>
<td>0.0086</td>
<td>8.0 mm</td>
<td>4.0 mm</td>
<td>2.0 : 1.0</td>
</tr>
<tr>
<td>7</td>
<td>0.0136</td>
<td>0.0086</td>
<td>4.75 mm</td>
<td>2.1 mm</td>
<td>2.3 : 1.0</td>
</tr>
<tr>
<td>8</td>
<td>0.0136</td>
<td>0.0089</td>
<td>3.75 mm</td>
<td>2.75 mm</td>
<td>1.4 : 1.0</td>
</tr>
<tr>
<td>9</td>
<td>pH 2.2</td>
<td>pH 2.1</td>
<td>4.5 mm</td>
<td>3.5 mm</td>
<td>1.3 : 1.0</td>
</tr>
<tr>
<td>10</td>
<td>pH 2.2</td>
<td>pH 2.1</td>
<td>2.15 mm</td>
<td>1.75 mm</td>
<td>1.3 : 1.0</td>
</tr>
<tr>
<td>11</td>
<td>&quot; 2.6&quot;</td>
<td>&quot; 2.6&quot;</td>
<td>1.65 mm</td>
<td>1.65 mm</td>
<td>1.0 : 1.0</td>
</tr>
<tr>
<td>12</td>
<td>&quot; 2.4&quot;</td>
<td>&quot; 2.4&quot;</td>
<td>2.0 mm</td>
<td>1.75 mm</td>
<td>1.1 : 1.0</td>
</tr>
<tr>
<td>13</td>
<td>&quot; 2.4&quot;</td>
<td>&quot; 2.4&quot;</td>
<td>1.7 mm</td>
<td>1.5 mm</td>
<td>1.1 : 1.0</td>
</tr>
<tr>
<td>14</td>
<td>&quot; 2.6&quot;</td>
<td>&quot; 2.6&quot;</td>
<td>2.15 mm</td>
<td>3.15 mm</td>
<td>0.68 : 1.0</td>
</tr>
<tr>
<td>15</td>
<td>&quot; 2.6&quot;</td>
<td>&quot; 2.6&quot;</td>
<td>1.50 mm</td>
<td>1.75 mm</td>
<td>0.85 : 1.0</td>
</tr>
<tr>
<td>16</td>
<td>&quot; 2.6&quot;</td>
<td>&quot; 2.6&quot;</td>
<td>1.35 mm</td>
<td>1.80 mm</td>
<td>0.75 : 1.0</td>
</tr>
<tr>
<td>17</td>
<td>&quot; 2.6&quot;</td>
<td>&quot; 2.6&quot;</td>
<td>4.0 mm</td>
<td>4.0 mm</td>
<td>1.0 : 1.0</td>
</tr>
<tr>
<td>18</td>
<td>&quot; 3.0&quot;</td>
<td>&quot; 3.0&quot;</td>
<td>0 mm</td>
<td>1.60 mm</td>
<td>0 : 1.6</td>
</tr>
<tr>
<td>19</td>
<td>&quot; 3.0&quot;</td>
<td>&quot; 3.0&quot;</td>
<td>0 mm</td>
<td>2.00 mm</td>
<td>0 : 2.0</td>
</tr>
<tr>
<td>20</td>
<td>&quot; 3.0&quot;</td>
<td>&quot; 3.0&quot;</td>
<td>0 mm</td>
<td>2.5 mm</td>
<td>0 : 2.5</td>
</tr>
<tr>
<td>21</td>
<td>&quot; 4.6&quot;</td>
<td>&quot; 4.6&quot;</td>
<td>0 mm</td>
<td>2.5 mm</td>
<td>0 : 2.5</td>
</tr>
<tr>
<td>22</td>
<td>&quot; 4.6&quot;</td>
<td>&quot; 4.6&quot;</td>
<td>0 mm</td>
<td>1.75 mm</td>
<td>0 : 1.75</td>
</tr>
<tr>
<td>23</td>
<td>pH 3.6</td>
<td>pH 3.6</td>
<td>0 mm</td>
<td>1.75 mm</td>
<td>0 : 1.75</td>
</tr>
<tr>
<td>24</td>
<td>pH 3.6</td>
<td>pH 3.6</td>
<td>0 mm</td>
<td>1.75 mm</td>
<td>0 : 1.75</td>
</tr>
<tr>
<td>25</td>
<td>pH 3.6</td>
<td>pH 3.6</td>
<td>0 mm</td>
<td>1.75 mm</td>
<td>0 : 1.75</td>
</tr>
<tr>
<td>26</td>
<td>pH 3.6</td>
<td>pH 3.6</td>
<td>0 mm</td>
<td>1.75 mm</td>
<td>0 : 1.75</td>
</tr>
<tr>
<td>27</td>
<td>0.0225</td>
<td>0.0225</td>
<td>0.25 mm</td>
<td>2.5 mm</td>
<td>1.0 : 10</td>
</tr>
<tr>
<td>28</td>
<td>0.0225</td>
<td>0.0225</td>
<td>0.15 mm</td>
<td>1.35 mm</td>
<td>1.0 : 9</td>
</tr>
</tbody>
</table>
Purification of Pepsin

TABLE II—Concluded.

<table>
<thead>
<tr>
<th>Determination No.</th>
<th>Normality Before.</th>
<th>Digestion.</th>
<th>Ratio</th>
<th>Duration</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>29</td>
<td>pH 4.6</td>
<td>mm.</td>
<td>mm.</td>
<td>0 : 0</td>
<td>6</td>
</tr>
<tr>
<td>30</td>
<td>pH 4.6</td>
<td>0</td>
<td>0</td>
<td>14</td>
<td>4.6</td>
</tr>
<tr>
<td>31</td>
<td>0.0225</td>
<td>0</td>
<td>0</td>
<td>24</td>
<td>1.65</td>
</tr>
</tbody>
</table>

Blank experiments for diffusion, no current.

- Calculated assuming complete ionization.
- Water used in side chambers.

consequently the digestion given by each is a close approximation of their relative activities. An equal volume of 0.2 per cent hydrochloric acid was added to the solutions and the peptic activity of these diluted solutions determined with Mett tubes, and expressed as mm. of albumen digested. Either the normality or pH of the pepsin solutions was determined before and after each series. The normality was determined by titration and the pH colorimetrically.

Experiment 1. Isoelectric Point of Preparation II.—In Determinations 1 to 17 (Table II) hydrochloric acid was used in making up the different pepsin solutions; in Determinations 18 to 21, lactic acid; in Nos. 22 to 24, acetic acid (approximately N/10); and in Nos. 25 and 26, acetic acid and sodium acetate (approximately 1 volume of N/10 acetic acid to 7 volumes of N/10 sodium acetate).

These results show a maximum cathodic migration at approximately pH 2. This migration diminishes with ascending pH until at pH 3 there is no cathodic migration. The isoelectric point of this preparation must therefore be in the neighborhood of pH 2.5.

Experiment 2. Isoelectric Point of Preparation III.—This experiment was carried out in exactly the same manner as Experiment 1. In Determinations 1 to 10 hydrochloric acid was used in making up the different pepsin solutions; in Nos. 11 and 12, dilute lactic acid; in Nos. 13 to 17, dilute acetic acid; in Nos. 18 to 22 approximately N/10 acetic acid and sodium acetate; in Nos. 23 and 24, N/10 hydrochloric acid with sodium acetate;
TABLE III.
Preparation III. Migration of Pepsin under the Influence of an Electric Current.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before.</td>
<td>After.</td>
<td>Cathode.</td>
<td>Anode.</td>
<td>mm.</td>
</tr>
<tr>
<td>1</td>
<td>0.050</td>
<td>0.050</td>
<td>1.75</td>
<td>2.0</td>
<td>0.87: 1.0</td>
</tr>
<tr>
<td>2</td>
<td>0.050</td>
<td>0.050</td>
<td>1.25</td>
<td>2.0</td>
<td>0.62: 1.0</td>
</tr>
<tr>
<td>3</td>
<td>0.048</td>
<td>0.048</td>
<td>2.0</td>
<td>2.75</td>
<td>0.72: 1.0</td>
</tr>
<tr>
<td>4</td>
<td>0.048</td>
<td>0.048</td>
<td>1.5</td>
<td>1.5</td>
<td>1.0: 1.0</td>
</tr>
<tr>
<td>5</td>
<td>0.012</td>
<td>0.011</td>
<td>2.0</td>
<td>2.5</td>
<td>0.80: 1.0</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td>2.0</td>
<td>3.0</td>
<td>0.69: 1.0</td>
</tr>
<tr>
<td>7</td>
<td>pH 2.0</td>
<td>pH 2.0</td>
<td>3.5</td>
<td>2.0</td>
<td>1.75: 1.0</td>
</tr>
<tr>
<td>8</td>
<td>&quot; 2.0 &quot;</td>
<td>&quot; 2.1 &quot;</td>
<td>4.5</td>
<td>2.5</td>
<td>1.8: 1.0</td>
</tr>
<tr>
<td>9</td>
<td>&quot; 2.0 &quot;</td>
<td>&quot; 2.0 &quot;</td>
<td>2.5</td>
<td>1.0</td>
<td>2.5: 1.0</td>
</tr>
<tr>
<td>10</td>
<td>&quot; 2.0 &quot;</td>
<td>&quot; 2.0 &quot;</td>
<td>2.5</td>
<td>1.0</td>
<td>2.5: 1.0</td>
</tr>
<tr>
<td>11</td>
<td>&quot; 2.5 &quot;</td>
<td>Trace.</td>
<td>Trace.</td>
<td>Trace.</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>&quot; 2.4 &quot;</td>
<td>Trace.</td>
<td>Trace.</td>
<td>Trace.</td>
<td></td>
</tr>
<tr>
<td>13†</td>
<td>&quot; 2.7 &quot;</td>
<td>0</td>
<td>4.0</td>
<td>0</td>
<td>0: 4.0</td>
</tr>
<tr>
<td>14</td>
<td>&quot; 2.6 &quot;</td>
<td>Trace.</td>
<td>Trace.</td>
<td>Trace.</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>&quot; 2.6 &quot;</td>
<td>&quot; 2.6 &quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>&quot; 2.7 &quot;</td>
<td>&quot; 2.6 &quot;</td>
<td>0.5</td>
<td>0.5</td>
<td>1.0: 1.0</td>
</tr>
<tr>
<td>17</td>
<td>&quot; 2.7 &quot;</td>
<td>&quot; 2.6 &quot;</td>
<td>0.5</td>
<td>0.5</td>
<td>1.0: 1.0</td>
</tr>
<tr>
<td>18</td>
<td>&quot; 3.0 &quot;</td>
<td>&quot; 3.0 &quot;</td>
<td>0</td>
<td>2.6</td>
<td>0: 2.6</td>
</tr>
<tr>
<td>19</td>
<td>&quot; 3.0 &quot;</td>
<td>&quot; 3.0 &quot;</td>
<td>0</td>
<td>1.75</td>
<td>0: 1.75</td>
</tr>
<tr>
<td>20</td>
<td>&quot; 3.0 &quot;</td>
<td>&quot; 3.0 &quot;</td>
<td>0</td>
<td>3.0</td>
<td>0: 3.0</td>
</tr>
<tr>
<td>21</td>
<td>&quot; 3.4 &quot;</td>
<td>&quot; 3.3 &quot;</td>
<td>0.5</td>
<td>3.0</td>
<td>1: 6.0</td>
</tr>
<tr>
<td>22</td>
<td>&quot; 3.4 &quot;</td>
<td>&quot; 3.4 &quot;</td>
<td>0</td>
<td>1.7</td>
<td>0: 1.7</td>
</tr>
<tr>
<td>23</td>
<td>&quot; 5.6 &quot;</td>
<td>&quot; 5.6 &quot;</td>
<td>0</td>
<td>1.75</td>
<td>0: 1.75</td>
</tr>
<tr>
<td>24</td>
<td>&quot; 5.6 &quot;</td>
<td>&quot; 5.6 &quot;</td>
<td>0</td>
<td>1.0</td>
<td>0: 1.0</td>
</tr>
<tr>
<td>25</td>
<td>&quot; 2.0 &quot;</td>
<td>&quot; 3.0 &quot;</td>
<td>0</td>
<td>3.5</td>
<td>0: 3.5</td>
</tr>
<tr>
<td>26</td>
<td>&quot; 2.0 &quot;</td>
<td>&quot; 3.0 &quot;</td>
<td>0</td>
<td>3.0</td>
<td>0: 3.0</td>
</tr>
</tbody>
</table>

* Calculated assuming complete ionization.
† This determination must be wrong because further determinations at the same pH gave different results, so it is not considered.

The isoelectric point exhibited by Preparation III is practically identical with that shown by Preparation II. The pepsin migrated solely towards the anode at pH 3 and above, and showed maximum cathodic migration at pH 2.

575
and in Nos. 25 and 26, hydrochloric acid. Dilute hydrochloric acid was used in the anode electrode for Determinations 1 to 17 inclusive. This was replaced in Determinations 18 to 26 by a 0.9 per cent sodium chloride solution which was slightly acidified with hydrochloric acid. In Determinations 25 and 26 the acid concentration of the anode electrode vessel was not sufficient to replace the loss in hydrogen ion sustained, through migration, by the central and side chambers. A considerable portion of the current was therefore carried by the sodium ions, thus resulting in the migration of the sodium ions to the central and side chambers with a corresponding decrease in the hydrogen ion concentration of these solutions. The pepsin migration manifested under such a condition would be determined by the minimum hydrogen ion concentration attained. The experimental results show this to be the case (Table III).

6. (a) Effect of Pepsin on Conductivity of Hydrochloric Acid Solutions.

A 0.4 per cent pepsin solution (Preparation III) in approximately 0.2 per cent hydrochloric acid was made up and left at room temperature for 4 hours. The conductivity of the solution was then compared with that of hydrochloric acid of the same concentration.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Specific conductivity of HCl (mhos)</th>
<th>Specific conductivity of pepsin solution (mhos)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.2</td>
<td>0.01491</td>
<td>0.01477</td>
</tr>
<tr>
<td></td>
<td>0.01477</td>
<td>0.01477</td>
</tr>
<tr>
<td></td>
<td>0.01477</td>
<td>0.01462</td>
</tr>
<tr>
<td></td>
<td>0.01477</td>
<td>0.01477</td>
</tr>
<tr>
<td></td>
<td>0.01480</td>
<td>0.01473</td>
</tr>
</tbody>
</table>

Average............. 0.01480 0.01473
The solutions were tightly corked and placed in an incubator at 37°C. for 5 hours and the conductivity redetermined:

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Specific conductivity of HCl (mhos)</th>
<th>Specific conductivity of pepsin solution (mhos)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0</td>
<td>0.01477</td>
<td>0.01456</td>
</tr>
<tr>
<td></td>
<td>0.01477</td>
<td>0.01435</td>
</tr>
<tr>
<td></td>
<td>0.01477</td>
<td>0.01456</td>
</tr>
<tr>
<td></td>
<td>0.01491</td>
<td>0.01435</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td><strong>0.01480</strong></td>
<td><strong>0.01445</strong></td>
</tr>
</tbody>
</table>

The solutions were tightly corked and placed in an incubator at 37°C. for 44 hours:

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Specific conductivity of HCl (mhos)</th>
<th>Specific conductivity of pepsin solution (mhos)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0</td>
<td>0.01477</td>
<td>0.01422</td>
</tr>
<tr>
<td></td>
<td>0.01462</td>
<td>0.01384</td>
</tr>
<tr>
<td></td>
<td>0.01491</td>
<td>0.01384</td>
</tr>
<tr>
<td></td>
<td>0.01477</td>
<td>0.01380</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td><strong>0.01477</strong></td>
<td><strong>0.01392</strong></td>
</tr>
</tbody>
</table>

Decrease in specific conductivity, 0.00085 mhos.

These results show that the conductivity of hydrochloric acid is only very slightly influenced by pepsin. However, if the pepsin solution is incubated for some time there is a slight but noticeable decrease in conductivity. This decrease in conductivity probably arises from one of two causes: firstly, the hydrolysis of protein impurities present in the pepsin preparation with the consequent liberation of free amino groups, which combine with the hydrochloric acid; secondly, the hydrolysis of the pepsin molecules themselves with the liberation of free amino groups. Unpublished experiments by the author show that when pepsin solutions are kept at 37°C. for even as short a time as 24 hours there is a noticeable decrease in their proteolytic activity, so it is probable that the second explanation is the correct one.
### TABLE IV.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>E.M.F. of pepsin solution</th>
<th>E.M.F. of HCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>309.5</td>
<td>309.7</td>
</tr>
<tr>
<td></td>
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<tr>
<td>pH</td>
<td>1.038</td>
<td>1.040</td>
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<tr>
<td>$H^+$</td>
<td>$9.16 \times 10^{-2}$</td>
<td>$9.12 \times 10^{-2}$</td>
</tr>
</tbody>
</table>

### TABLE V.

Determination after 6 hrs. at room temperature.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>E.M.F. of pepsin solution</th>
<th>E.M.F. of HCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>325.3</td>
<td>325.3</td>
</tr>
<tr>
<td></td>
<td>325.2</td>
<td>325.3</td>
</tr>
<tr>
<td></td>
<td>325.1</td>
<td>325.4</td>
</tr>
<tr>
<td></td>
<td>325.3</td>
<td>325.4</td>
</tr>
<tr>
<td></td>
<td>325.3</td>
<td>325.3</td>
</tr>
<tr>
<td>Average</td>
<td>325.2</td>
<td>325.35</td>
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<tr>
<td>pH</td>
<td>1.314</td>
<td>1.317</td>
</tr>
<tr>
<td>$H^+$</td>
<td>$4.85 \times 10^{-2}$</td>
<td>$4.82 \times 10^{-2}$</td>
</tr>
</tbody>
</table>

Determination after 15 hrs. at 37°C.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>E.M.F. of pepsin solution</th>
<th>E.M.F. of HCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>324.5</td>
<td>325.3</td>
</tr>
<tr>
<td></td>
<td>324.5</td>
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<td>325.4</td>
</tr>
<tr>
<td></td>
<td>324.8</td>
<td>325.4</td>
</tr>
<tr>
<td>Average</td>
<td>324.6</td>
<td>325.35</td>
</tr>
<tr>
<td>pH</td>
<td>1.303</td>
<td>1.317</td>
</tr>
<tr>
<td>$H^+$</td>
<td>$4.98 \times 10^{-2}$</td>
<td>$4.82 \times 10^{-2}$</td>
</tr>
</tbody>
</table>

### TABLE VI.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>E.M.F. of pepsin solution</th>
<th>E.M.F. of HCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>326.5</td>
<td>325.3</td>
</tr>
<tr>
<td></td>
<td>326.5</td>
<td>325.4</td>
</tr>
<tr>
<td></td>
<td>326.4</td>
<td>325.3</td>
</tr>
<tr>
<td></td>
<td>326.4</td>
<td>325.4</td>
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<tr>
<td>Average</td>
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<td>325.35</td>
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<tr>
<td>pH</td>
<td>1.336</td>
<td>1.317</td>
</tr>
<tr>
<td>$H^+$</td>
<td>$4.61 \times 10^{-2}$</td>
<td>$4.82 \times 10^{-2}$</td>
</tr>
</tbody>
</table>

Decrease in $H^+$, $1.9 \times 10^{-3}$
(b) Effect of Pepsin on Hydrogen Ion Concentrations of Acid Solutions.

Purified pepsin (Preparation III) was dissolved in dilute hydrochloric acid and the hydrogen ion concentration of this solution compared with that of the hydrochloric acid itself. A Leeds and Northrup hydrogen ion apparatus, with a saturated potassium chloride calomel electrode, was used in all these determinations.

Experiment 1.—0.0900 gm. of purified pepsin was dissolved in 15 cc. of acid and put aside at room temperature for about 48 hours. The results are given in Table IV.

Experiment 2.—0.2000 gm. of purified pepsin was dissolved in 50 cc. of approximately 0.2 per cent hydrochloric acid and the hydrogen ion concentration of it compared with that of the stock hydrochloric acid after 6 hours at room temperature. The results are given in Table V.

Experiment 3.—0.1000 gm. of purified pepsin was dissolved in 25 cc. of acid and the solution incubated at 37°C. for 44 hours. The experimental results, given in Table VI, show that pepsin has no noticeable influence on the hydrogen ion concentration of acid solutions. If the solutions, however, are kept at 37°C. for some time there is a small but noticeable decrease in the hydrogen ion concentration.

7. Effect of Ether Extraction on Activity of Purified Pepsin.

Experiment 1.—About 0.1 gm. of pepsin was placed in an extraction thimble and extracted with ether in a Soxhlet apparatus for 2½ hours. The activity of the residue was then compared with that of the original sample by the Mett tube method.

<table>
<thead>
<tr>
<th>Original material</th>
<th>Extracted material</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight in 10 cc. acid</td>
<td>0.0075 gm.</td>
</tr>
<tr>
<td>Digestion</td>
<td>5.0 mm.</td>
</tr>
<tr>
<td>Comparative digestions (calculated)</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Experiment 2.—This experiment was carried out in exactly the same manner as Experiment 1, except that the extraction was continued for 5 hours.
Purification of Pepsin

Weight in 10 cc. acid.......................... 0.0060 gm. 0.0075 gm.
Digestion........................................ 6.75 mm. 8.0 mm.
Comparative digestion (calculated)........ 7.5 " 8.0 "

The above results show that extraction with ether does not affect the proteolytic activity of pepsin.

TABLE VII.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cc.</td>
<td>cc.</td>
<td>cc.</td>
<td>cc.</td>
<td>mm.</td>
<td>mm.</td>
<td>mm.</td>
</tr>
<tr>
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<td>10</td>
<td>5</td>
<td>22</td>
<td>5.0</td>
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<tr>
<td>2</td>
<td>18</td>
<td>9</td>
<td>6</td>
<td>20</td>
<td>5.4</td>
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<td>10</td>
<td>20</td>
<td>5.0</td>
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</tr>
</tbody>
</table>

TABLE VIII.

<table>
<thead>
<tr>
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<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
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<tr>
<td></td>
<td>cc.</td>
<td>cc.</td>
<td>cc.</td>
<td>cc.</td>
<td>mm.</td>
<td>mm.</td>
<td>mm.</td>
<td>Standard. Filtrate.</td>
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<tr>
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<td>5</td>
<td>32</td>
<td>7.5</td>
<td>6.2</td>
<td>0</td>
<td>7.5 7.5</td>
</tr>
<tr>
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<td>60</td>
<td>25</td>
<td>20</td>
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<td>9.5</td>
<td>10.0</td>
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<td>17</td>
<td>9.5</td>
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<td>10</td>
<td>10</td>
<td>10</td>
<td>10.0</td>
<td>8.0</td>
<td>8.0</td>
<td>10.0 8.0</td>
</tr>
</tbody>
</table>

8. Effect of Alcohol and Ether on Pepsin Solutions in Various Acid Concentrations.

During the adsorption experiments with cholesterol it was found that if the cholesterol-alcohol-ether solution was added to an acid solution of pepsin the enzyme was almost completely inactivated. In order to ascertain the cause of this inactivation, it was decided to determine the effect of an alcohol-ether mixture on solutions of pepsin in various acid concentrations. Experiments were therefore carried out, using hydrochloric and phosphoric acid solutions of pepsin.
Experiment 1.—Purified pepsin was dissolved in 0.2 per cent hydrochloric acid and various amounts of alcohol and ether added. If a precipitate formed it was filtered off and the filtrate placed in an evaporating dish and left until the odor of ether had disappeared. The activity of the solution was then compared with that of the original untreated pepsin solution (standard). The portion of the filter paper containing the precipitate was put into 10 cc. of 0.2 per cent hydrochloric acid, Mett tubes added, and the digestive power determined in the usual manner. The results are given in Table VII.

Experiment 2.—Same as above except that the pepsin solutions were previously dialyzed against distilled water. pH about 3.5. Table VIII shows the results.

Experiment 3.—Purified pepsin was dissolved in phosphoric acid solutions of various concentrations and treated in the same manner as in the previous experiments. The results are given in Table IX.

<table>
<thead>
<tr>
<th>Determination No.</th>
<th>Normality</th>
<th>Solution taken</th>
<th>Alcohol added</th>
<th>Ether added</th>
<th>Volume after evaporation</th>
<th>Digestion filtrate</th>
<th>Digestion precipitate</th>
<th>Comparative digestion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cc.</td>
<td>cc.</td>
<td>cc.</td>
<td>cc.</td>
<td>mm.</td>
<td>mm.</td>
<td>mm.</td>
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</tr>
<tr>
<td>1</td>
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<td>10</td>
<td>10</td>
<td>5</td>
<td>14</td>
<td>4.7</td>
<td>1.0</td>
<td>4.7</td>
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<td>2</td>
<td>0.685</td>
<td>10</td>
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<td>5</td>
<td>7.5</td>
<td>4.6</td>
<td>1.5</td>
<td>4.6</td>
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<tr>
<td>3</td>
<td>0.685</td>
<td>10</td>
<td>10</td>
<td>5</td>
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<td>0</td>
<td>4.7</td>
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<td>10</td>
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<tr>
<td>8</td>
<td>0.342</td>
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<td>5</td>
<td>11</td>
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<td>11</td>
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<td>4.6</td>
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<tr>
<td>12</td>
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<td>4.7</td>
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<td>3.3</td>
<td>3.0</td>
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<td>0</td>
<td>21</td>
<td>4.7</td>
<td>2.7</td>
<td>3.0</td>
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</table>
These results show that the enzyme is readily destroyed at moderately high hydrogen ion concentrations by alcohol-ether mixture, but that it is not affected in solutions of low hydrogen ion concentrations. Mixtures of ether and alcohol also appear to exert a greater destructive reaction than alcohol itself.

DISCUSSION.

Adsorption of pepsin with aluminium hydroxide, dissolving the hydroxide with dilute acid, and precipitating the pepsin by dialysis gave quite an active preparation, but further attempts at its purification by solution in acid and reprecipitation by dialysis were unsuccessful, a great amount of the active principle being lost during the treatment. The safranine precipitation method, on the other hand, gave a very active preparation. This method of purification has a great advantage over all previous methods employed in the purification of pepsin, for safranine does not precipitate much, if any, of the accompanying protein impurities. This is evident from the fact that, although the active principle is quantitatively precipitated from solutions of commercial pepsin, yet the weight of the precipitate is only about 5 per cent of the material taken. The usual methods of purification by precipitation with protein precipitants such as sodium chloride, ammonium sulfate, basic and neutral lead acetate, must yield products containing considerable proteins. Even precipitation by dialysis, as carried out by Pekelharing and by the writer, apparently result in the precipitation of some of the protein impurities as well as the pepsin. This is supported by the fact that the preparations obtained during this investigation by adsorption with aluminium hydroxide and subsequent dialysis were only about 70 per cent as active as those obtained by the safranine precipitation method. A comparison of the results of the chemical analysis of these various preparations with those obtained from typical animal proteins lends additional support to the above hypothesis. The greater the degree of purification the less the analysis resembles that of typical proteins.
The isoelectric point of two pepsin preparations was determined and found to be pH 2.5. This agrees fairly well with Michaelis and Davidsohn’s value of pH 2.26. These results, as well as the fact that safranine, a basic dye, quantitatively precipitates the active principle, are strong evidence in favor of the assumption that the enzyme possesses very marked acidic characteristics. The basic characteristics must consequently be very low. That this is probably so is evident from the fact that on the acid side of the isoelectric point, where basic characteristics should be most evident, there is no noticeable decrease either in the hydrogen ion concentration or in the conductivity of an acid solution after addition of purified pepsin. Further evidence also in favor of the above assumption is the low percentage of the total nitrogen present as hexone base nitrogen; that is, as arginine, histidine, and lysine nitrogen. These amino acids must be responsible for most of the basic characters of protein. Therefore, since only 7.44 per cent of the total nitrogen in Preparation II was present as hexone base nitrogen, it is evident that its basic characters must be correspondingly low.

The effect of a mixture of equal amounts of alcohol and ether on pepsin in solution was found to depend to a great extent on the hydrogen ion concentration of the solution, the enzyme being inactivated in solutions of high acidity while its activity was unaffected in solutions of low hydrogen ion concentration. The extraction of solid pepsin with ether, on the other hand, does not affect the proteolytic activity of the enzyme.

**SUMMARY.**

1. The adsorption of pepsin by animal charcoal, Lloyd’s reagent, kaolin, salicylic acid, acetanilide, cholesterol, camphor,
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aluminium oxide, and freshly precipitated aluminium hydroxide, was studied and found to vary greatly for the different adsorbents.

2. Pepsin preparations were obtained by different methods and analyzed. The most active preparation was obtained by precipitating the pepsin with safranine and removing the safranine with isoamyl alcohol. A comparison of the various analyses with those from typical proteins shows that the greater the purity of the preparation the less its composition resembles that of typical proteins.

3. Purified pepsin was hydrolyzed and a number of amino acids isolated from the hydrolytic products with infinitesimal traces of purine bases.

4. The nitrogen distribution of the products of hydrolysis was determined according to Andersen and Roed-Müller's method. In these products monoamino acid nitrogen greatly preponderated.

5. The isoelectric point of two different preparations was determined and found to be pH 2.5.

6. Determinations were carried out on the effect of pepsin on the hydrogen ion concentration and conductivity of acid solutions. The results show that purified pepsin has no immediate appreciable effect on either of the properties. However, if the solution was incubated for some time a small but definite decrease was obtained, both in conductivity and hydrogen ion concentration.

7. Various preparations of pepsin were extracted with ether in a Soxhlet apparatus. The activity of the preparation was not affected by this treatment.

8. The effect of adding mixtures of alcohol and ether to solutions of pepsin of various hydrogen ion concentrations was studied. The results showed that the enzyme was rapidly inactivated in solutions of moderately high hydrogen ion concentrations by alcohol-ether mixtures but that it was unaffected in solutions of low hydrogen ion concentrations.

In conclusion, I wish to express my thanks to the Research Council of Canada, who made this investigation possible by the grant of several studentships to the author, and to Professor A. B. Macallum, under whom this research was carried on, for his kind direction and advice.
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THE PURIFICATION OF PEPSSIN, ITS PROPERTIES, AND PHYSICAL CHARACTERS
J. C. Forbes


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