ON THE INCREASE IN WEIGHT IN THE HYDROLYSIS OF CASEIN.

BY J. H. LONG.

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In the hydrolysis of proteins accomplished by the action of trypsin and alkali, or pepsin and acid there is, as is well known, a considerable increase in weight due to the absorption of water, or water and hydrochloric acid. There are but few references in the literature to the extent of this weight alteration or to the stability of the products formed.

In some recent work on the digestion of casein I have called attention to the amount of the evaporation residue from liquids left after prolonged digestion, and in the following I shall give some results obtained in a series of experiments running through several weeks, in which casein was digested with acid and pepsin.

In these experiments the casein used was made from skimmed cows' milk by the Hammarsten process, and was finally air dried. It left but a minute trace of ash on ignition and was readily soluble in weak alkali giving a nearly clear solution. In the air dry condition, as used, it contained 4 percent of moisture. The pepsin employed was a specially active product, referred to in the paper cited above. 2 grams of this pepsin were dissolved in 2000 cc. of weak hydrochloric acid containing in each cubic centimeter 2.63 mg. of actual HCl. On evaporating 50 cc. of this mixture to dryness on the water-bath and keeping at a temperature of 102° through half an hour in the air oven a residue weighing 53 milligrams was obtained, showing a slight increase in weight from addition of water, or hydrochloric acid, or both.

In the digestion experiments I charged each of 8 small flasks with 1.5 gms. of the casein, and 150 cc. of the pepsin-acid mixture. The flasks were closed with rubber stoppers, holding glass tubes with fine capillary openings, and were immersed in a water reservoir maintained at a temperature of 40° through the time of the tests. The capillary tubes extended above the surface of the water and served to maintain the atmospheric pressure in the flasks, while preventing any appreciable evaporation of the digesting mixture, which had a volume of about 151 cc. This volume remained practically constant through the tests.

In mixing acid with casein and titrating immediately, the whole of the acid is shown when phenolphthalein is used but not when methyl orange or the related substance dimethylaminoazobenzene is employed. This fact was illustrated by the results obtained with a ninth flask charged exactly as were the others described. On withdrawing 50.3 cc. and titrating at once 32.6 cc. of alkali were required with the methyl orange indicator and 36.0 cc. with the phenolphthalein. If the titration is not...
made rapidly there may be a little excess of alkali needed in the second
test through combination of the casein itself which behaves as an acid.

The eight flasks were put in the thermostat on the morning of Nov.
20th and from time to time one was removed for examination, as shown
in the table below. For each test one-third of the total volume was
taken, which was practically 50.3 cc. This corresponds to 500 mg. of
the original casein, or 480 mg. of dry casein. A direct titration was
made using dimethylaminoazobenzene and phenolphthalein. A second
portion of 50.3 cc. was evaporated to dryness in platinum at a low tem-
perature and then dried in 102° in the air oven. The residue was weighed
and from the weight found that of the pepsin residue spoken of above,
was subtracted. This, as shown, was 53 mg. After weighing the res-
idue in the platinum dish it was moistened with a little sodium car-
bonate solution, evaporated again and ignited. In the ash the chlorine
was found and this was calculated to HCl. The results are given in the
table. Direct experiments showed that the amount of chlorine in the
pepsin used was but a trace and not large enough to show in the final re-

sult.

In a number of cases the third portion of the digestion mixture was
used for titration with $p$-nitrophenol, which appears to react with the
hydrochloric acid, free and combined. In all cases N/10 sodium hydroxide
was used in the titrations.

<table>
<thead>
<tr>
<th>No.</th>
<th>Date</th>
<th>Dry Casein</th>
<th>Dimethyl-</th>
<th>Phenol-</th>
<th>Dry residue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>aminoazo</td>
<td>phthalein</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>benzene</td>
<td>titration</td>
<td>titration</td>
</tr>
<tr>
<td>1</td>
<td>Nov. 21</td>
<td>480 mg.</td>
<td>31 cc.</td>
<td>44 cc.</td>
<td>36.0 cc.</td>
</tr>
<tr>
<td>2</td>
<td>22</td>
<td>480</td>
<td>30.5</td>
<td>44.8</td>
<td>612</td>
</tr>
<tr>
<td>3</td>
<td>26</td>
<td>480</td>
<td>29.5</td>
<td>45.2</td>
<td>651</td>
</tr>
<tr>
<td>4</td>
<td>30</td>
<td>480</td>
<td>28.5</td>
<td>45.5</td>
<td>651</td>
</tr>
<tr>
<td>5</td>
<td>Dec. 5</td>
<td>480</td>
<td>28.0</td>
<td>45.5</td>
<td>661</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>480</td>
<td>27.0</td>
<td>45.5</td>
<td>659</td>
</tr>
<tr>
<td>7</td>
<td>15</td>
<td>480</td>
<td>27.0</td>
<td>45.5</td>
<td>657</td>
</tr>
<tr>
<td>8</td>
<td>22</td>
<td>480</td>
<td>26.5</td>
<td>45.6</td>
<td>660</td>
</tr>
</tbody>
</table>

It will be observed that the weight increase is divided irregularly
between the water and hydrochloric acid. The results found vary some-
what with the duration of the drying at the end of the evaporation, as
one experiment showed. The remaining third of the liquid in flask 3
was evaporated to dryness on the water-bath, kept there one hour and
then weighed. Later weighings were made after heating for different per-
iods, as follows, the results being diminished in each case by 53 mg. on
account of the pepsin residue.

First weight........................................... 0.606
After heating $\frac{1}{2}$ hour to 105°...................... 0.587
After 1 hour more to 105°.......................... 0.584
After 4 hours more to 105°........................ 0.581
The residue was then ashed and the chlorine content found. Calculated as HCl it amounted to 86 mg.

It would appear from this that the hydrolysis and salt products are relatively stable, and also that there is more danger of losing water than hydrochloric acid by prolonged heating. It must be recognized, of course, that much of this addition of water and acid follows in the final evaporation rather than in the prolonged digestion at 40°. This is certainly true as far as the combination with hydrochloric acid is concerned, if we can depend on the information given by the dimethylaminoazobenzene titration. We have no direct means of determining the amount of water added in the digestion stage, but some information may be derived from the results of the titration with phenolphthalein. After the first twenty-four hours of digestion, there is not much change in the total acidity as measured by the phenolphthalein, but as compared with the original acid value and the P-nitrophenol titration there is an increase of 9.5 cc. of N/10 alkali required in the titration. This corresponds to the acids of the amino type formed in the reaction and in the production of such acids a certain amount of water must be added to hydrolyze the more complex parent groups. A small portion of this excess of alkali may be used in another way as will be explained below. As casein is known to yield a large amount of glutaminic acid in complete hydrolysis, let us assume for illustration that it consists of a number of such acid groups, built up in polypeptide form. The hexone bases, known to be relatively abundant hydrolysis products of casein might be taken just as well, but for simplicity the following typical arrangement may be assumed:

\[ -\text{NHCHCO-} \quad -\text{NHCHCO-} \quad -\text{NHCHCO-} \]

\[ C_4H_8O_2 \quad C_4H_8O_2 \quad C_4H_8O_2 \]

The addition of \( n \) molecules of water to such a complex would yield \( n \) molecules of glutaminic acid, each one of which would require one or two molecules of alkali for neutralization, depending on the character of the indicator used. In other words, for each molecule of acid formed we must calculate one molecule of water added, and in the above illustration an increase in weight from 129 to 147. In titration, however, using phenolphthalein, only one carboxyl group appears to act and the salts formed are of the type \( C_6H_5NO_3Na \). Some direct titration experiments with glutaminic acid which I prepared from casein, gave approximately this result, but were not wholly satisfactory because of the presence of other acids not well removed in the purification. With the closely related aspartic acid the phenolphthalein titration was sharp, with the formation of a salt of the formula \( C_4H_8NO_4Na \). In this case 1 cc. of \( N/10 \) alkali corresponds to 1.8 mg. of added water, and the 9.5 cc. of excess alkali, therefore to 17.1 mg. of water. As some of the amino acids
act very feebly toward alkali and phenolphthalein, it is likely that in
the mean, considering the various acids which may be formed, 1 cc. of
the N/10 alkali would correspond to even more than 1.8 mg. of water.
For the purpose of comparison, however, we may assume this value in
the calculations below. At the same time we will assume that the reac-
tion with dimethylaminoazobenzene measures the free hydrochloric acid,
and thus reach the fraction of this body combined during the preliminary
digestion. It must be said, however, that the delicacy of this indicator
for the purpose is generally overrated.

In the table below are given some figures calculated in part from the
data of the first table and in part according to the assumptions just made
concerning the water and the hydrochloric acid.

<table>
<thead>
<tr>
<th>No.</th>
<th>H₂O added in</th>
<th>H₂O added,</th>
<th>HCl added in</th>
<th>HCl added,</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>digestion</td>
<td>total</td>
<td>digestion</td>
<td>total</td>
</tr>
<tr>
<td>1</td>
<td>14.4</td>
<td>16.0</td>
<td>18.3</td>
<td>94.9</td>
</tr>
<tr>
<td>2</td>
<td>15.8</td>
<td>33.0</td>
<td>20.1</td>
<td>99.0</td>
</tr>
<tr>
<td>3</td>
<td>16.6</td>
<td>49.0</td>
<td>23.7</td>
<td>102.2</td>
</tr>
<tr>
<td>4</td>
<td>17.1</td>
<td>67.0</td>
<td>27.4</td>
<td>104.0</td>
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<td>17.1</td>
<td>74.0</td>
<td>29.2</td>
<td>107.1</td>
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<td>72.0</td>
<td>32.8</td>
<td>107.1</td>
</tr>
<tr>
<td>7</td>
<td>17.1</td>
<td>65.0</td>
<td>32.8</td>
<td>112.0</td>
</tr>
<tr>
<td>8</td>
<td>17.3</td>
<td>68.7</td>
<td>34.7</td>
<td>111.3</td>
</tr>
</tbody>
</table>

It appears from this method of calculation that the water which is
added in the digestion does not vary much after the first periods; the
hydrochloric acid is added more slowly and shows a gradual increase. On
the other hand, in the final evaporation periods there is a marked
increase in the added water in the later flasks, and a relatively small in-
crease in the added hydrochloric acid.

It is interesting to note the relations which exist between the weight
of dry casein in each flask, 480 mg., and the final weight of water and acid
added. Toward the end of the experiments we have, in the mean, about
70 mg. of added water and 110 mg. of added acid. If we assume for
illustration, as was done above, that a forerunner of the glutaminic acid
complex undergoes hydration we must give this primary complex a mole-
cular weight lower by 18 than that of the finished acid. For glutaminic
acid this would give us 147 - 18 = 129, and the increase in weight for
480 mg. would be shown in this way:

\[
\frac{129}{18} = \frac{480}{x} \quad \therefore x = 67
\]

\[
\frac{129}{36.5} = \frac{480}{x} \quad \therefore x = 135.8
\]

The actual conditions cannot be as simple as here assumed for illus-
tration, but it is evident that the final changes correspond to rather com-
plete hydrolysis and salt formation. The digestive mixtures which were
colourless at the start became slightly brown in the last ten days of the
experiments, indicating an advanced degree of hydrolysis.
It will be recalled that casein itself combines rather readily with alka-
lies and I have pointed out elsewhere¹ that 1 gm. of pure casein may be
combined with 9 cc. of N/10 alkali to form a 'neutral' compound or
with 4.5 cc. to form what may be considered as an acid salt. In forming
the first, or so-called neutral casein, there is apparently some hydrolysis,
as the whole of the substance can not be completely recovered by addi-
tion of acetic acid. The 'acid' salt is soluble, and may be formed from
carboxyl groups in the original casein. From this point of view a small
part of the excess of alkali used in titrating with phenolphthalein may
be required for carboxyl groups of the casein complex itself rather than
for similar groups of the amino acid formed. This would make the
water added in the actual digestion appear still smaller in amount, but I
have not tried to allow for this possible condition in the tables calculated.

It is of course not possible to define the extent of the hydrolysis at 40°
very closely, when it is brought about by weak acid and pepsin, but the
above experiments offer another proof that it must go far beyond the sim-
ple albumose formation of the older physiologists, if indeed further
proof on the question could be considered necessary. On this point see
the convenient literature resumé of Cohnheim.²

In referring to glutaminic acid it must be remembered that I take this
complex merely to illustrate the changes which would follow by water
addition. The same principles would naturally hold for much larger
groups, but the sharpness and directness of the titrations speak for the
formation of bodies of pronounced acid character. Another point also is
interesting to note here; this sharpness in the phenolphthalein titration
does not increase as the digestion progresses, but on the contrary seems
to grow less distinct. The tests made at the end of four weeks are not
as clear as those made at the end of two weeks. The final color reaction
with phenolphthalein reminds one of titrations in presence of traces of
ammonium salts, and here evidently points to the accumulation of amino
compounds which show an analogous behavior.

Finally attention must be called to the behavior of p-nitrophenol used
in some of the titrations. This indicator has not usually been considered
as very delicate, but in the estimation of total mineral acids in digestive
experiments it seems to have a place and may be found extremely useful.
Further experiments on this point are in progress.

¹ This Journal, 27, 363 and 28, 372.
² Cohnheim, Chemie der Eiweisskörper, 2d ed., p. 93.