ON THE INCREASE IN WEIGHT IN THE HYDROLYSIS OF CASEIN[1].

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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[2] 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

[1] Read at the New York meeting of the Am. Chemical Society, Dec. 31, 1906.

[2] This Journal, 29, 223.

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 temperature 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 residue in the platinum dish it was moistened with a little sodium carbonate 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 result.

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 I. DIRECT RESULTS.

No.	Date	Dry Casein	Dimethyl-aminoazo benzene titration	Phenol-phthalein titration	p-Nitrophenol titration	Dry residue Cor. (—53 mg.)	HCl in residue
1	Nov. 21	480 mg.	31 cc.	44 cc.	36.0 cc.	591 mg.	94.9 mg.
2	22	480	30.5	44.8		612	99.0
3	26	480	29.5	45.2		631	102.2
4	30	480	28.5	45.5		651	104.0
5	Dec. 5	480	28.0	45.5	36.0	661	107.1
6	10	480	27.0	45.5	36.0	659	107.1
7	15	480	27.0	45.5	36.0	657	112.0
8	22	480	26.5	45.6	36.0	660	111.3

It will be observed that the weight increase is divided irregularly between the water and hydrochloric acid. The results found vary somewhat 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 periods, as follows, the results being diminished in each case by 53 mg. on account of the pepsin residue.

First weight	0.606
After heating ½ 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:

$$\begin{array}{ccc} \text{—NHCHCO—} & \text{—NHCHCO—} & \text{—NHCHCO—} \\ \quad\vert\quad & \quad\vert\quad & \quad\vert\quad \\ \quad C_3H_5O_2 \quad & \quad C_3H_5O_2 \quad & \quad C_3H_5O_2 \end{array}$$

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 $C_5H_8NO_4Na$. 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 $C_4H_6NO_4Na$. 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 reaction 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 II. ADDITION OF H_2O AND HCl.

No.	H_2O added in digestion	H_2O added, total	HCl added in digestion	HCl added, total
1	14.4	16.0	18.3	94.9
2	15.8	33.0	20.1	99.0
3	16.6	49.0	23.7	102.2
4	17.1	67.0	27.4	104.0
5	17.1	74.0	29.2	107.1
6	17.1	72.0	32.8	107.1
7	17.1	65.0	32.8	112.0
8	17.3	68.7	34.7	111.3

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 increase 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 molecular 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:

$$129 : 18 :: 480 : x \cdot x = 67$$
$$129 : 36.5 :: 480 : x \cdot x = 135.8$$

The actual conditions cannot be as simple as here assumed for illustration, but it is evident that the final changes correspond to rather complete hydrolysis and salt formation. The digestive mixtures which were colorless 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 alkalies and I have pointed out elsewhere[1] 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 addition 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 simple 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.

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