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# EFFECT OF TIME OF DIGESTION ON THE HYDROLYSIS OF CASEIN IN THE PRESENCE OF STARCH[1]

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The Van Slyke[2] method for protein analysis was worked out upon mixtures of relatively pure amino acids and was not intended to be applied directly to crude sources of protein contained in cereals and feeding stuffs.

Notwithstanding this fact, Grindley, Slater, et al.,[3] of the Illinois Experiment Station, published in 1915 the results of the determination of the amino acids contained in cottonseed meal, tankage, and alfalfa hay, applying the Van Slyke method directly to the proteins contained in these different feeds.

In the same month of 1915 Nollau,[4] of this Station, published his results, obtained by the Van Slyke method, on about 25 different sources of crude protein contained in various seeds and feeding stuffs.

In December, 1915, Grindley, Slater, et al.,[3] published a second paper on the amino-acid content of various feeds, including wheat, oats, barley, and sov beans, a number of which had been analyzed by Nollau. The summary of their second paper in part is as follows:

> The results here reported confirm the conclusion previously drawn, namely, that the Van Slyke method for the determination of the chemical groups characteristic of the amino acids of proteins can be applied directly to the quantitative determinations of the amino acids of feeding stuffs with at least a fair degree of accuracy.
>
> The results which we have obtained for the quantitative determinations of amino acids in feeding stuffs, on the whole, do not agree well with those recently published by Nollau. In some determinations the results from the two sources are quite satisfactory, but in many cases the agreement is far from satisfactory. The lack of con-

[1] Approved for publication in the Journal of Agricultural Research by A. M. Peter, Acting Director, Kentucky Agricultural Experiment Station.

[2] VAN SLYKE, D. D. THE ANALYSIS OF PROTEINS BY DETERMINATION OF THE CHEMICAL GROUPS CHARACTERISTIC OF THE DIFFERENT AMINO ACIDS. *In* Jour. Biol. Chem., v. 10, no. 1, p. 15–55, 2 fig. 1911.

[3] GRINDLEY, H. S., SLATER, M. E., et al. THE QUANTITATIVE DETERMINATION OF THE AMINO ACIDS OF FEEDING STUFFS BY THE VAN SLYKE METHOD. *In* Jour. Amer. Chem. Soc., v. 37, no. 7, p. 1778–1781; no. 12, p. 2762–2769. 1915.

[4] NOLLAU, E. H. THE AMINO-ACID CONTENT OF CERTAIN COMMERCIAL FEEDING STUFFS AND OTHER SOURCES OF PROTEIN. *In* Jour. Biol. Chem., v. 21, no. 3, p. 611–614. 1915.

cordant results is probably due in the main to differences in the details of procedure in the experimental work.

Hart and Bentley,[1] of the Wisconsin Experiment Station, comment unfavorably on the lack of agreement between the results obtained by Grindley, Slater, et al., and those obtained by Nollau for the amount of the different amino-acid groups contained in feeding stuffs. They state that whether accurate determinations of any or all the amino acids can be secured when the hydrolyzing proteins are in contact with hydrolyzing carbohydrates must first be determined before these data can be accepted as final.

Presumably in order to substantiate the theory in regard to the effect of hydrolyzing carbohydrates on the different amino-acid groups in proteins, Hart and Sure [2] have published results obtained upon the hydrolysis of casein, alone and in the presence of a number of different carbohydrates. In one of their experiments, 2.4 gm. of casein and 12 gm. of starch were hydrolyzed by boiling in 20 per cent hydrochloric acid for a period of 48 hours. The result obtained for lysin in this experiment shows that approximately 50 per cent of this amino-acid group has been changed to some other form of combination. They summarize their results in part as follows:

> The Van Slyke method of protein analysis, applied to casein, hydrolyzed in the presence of various carbohydrates, brings about a total redistribution of the amino-acids varying with the nature of the carbohydrate employed. This work on casein and Gortner's work on fibrin, hydrolyzed in the presence of cellulose, *definitely* show the inapplicability of the method of direct hydrolysis for the estimation of amino-acids in feeding stuffs by Van Slyke's method. The results so secured will be inaccurate.

Upon the publication of Hart and Sure's results, it appeared to the writer that their conclusions were much broader than their experiments justified. In fact, Hart and Bentley [3] make statements which appear to be merely forecastings rather than conclusions arrived at by experimentation. In order to be able to say positively that the Van Slyke method for protein analysis can not be applied directly to heterogeneous mixtures of protein and carbohydrate requires much further experimentation. It is by no means to be taken for granted that results obtained on a 48-hour digestion will be the same as those carried on for a shorter length of time.

It therefore occurred to the writer that a duplication of the experiment of Hart and Sure upon the effect produced on the hydrolysis of casein by the presence of starch, in which the time of digestion varied, would afford more conclusive evidence on this subject. Accordingly, five experiments were planned, as follows:

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[1] HART, E. B., and BENTLEY, W. H. THE CHARACTER OF THE WATER-SOLUBLE NITROGEN OF SOME COMMON FEEDING STUFFS. *In* Jour. Biol. Chem., v. 22, no. 3, p. 477–483. 1915.

[2] ..... and SURE, Barnett. THE INFLUENCE OF CARBOHYDRATES ON THE ACCURACY OF THE VAN SLYKE METHOD IN THE HYDROLYSIS OF CASEIN. *In* Jour. Biol. Chem., v. 28, no. 1, p. 241–249. 1916.

[3] HART, E. B., and BENTLEY, W. H. Op. cit.

Five 10-gm. portions of Hammarsten's casein were weighed out and transferred to five 1-liter round-bottom Jena flasks. Fifty gm. of cornstarch were then weighed out and added to each of the flasks except the first, which contained casein alone. Three hundred c. c. of 20 per cent hydrochloric acid, specific gravity 1.11, were added to each flask. All the flasks were then heated on the water bath, with frequent shakings, for about two hours. The object of this preliminary heating on the water bath was to liquefy the starch-casein mixtures, which had gelatinized upon the addition of the hydrochloric acid. After the starch had become liquid all the flasks were removed and attached to reflux condensers and heated to a gentle boil.

Experiments 1 and 2 were allowed to digest for 12 hours, No. 3 for 15 hours, No. 4 for 24 hours, and No. 5 for 48 hours, each being cut out at the expiration of its time interval.

After each of the experiments had stood at room temperature for six or eight hours, they were filtered through paper on a Buchner funnel and washed practically free of chlorids with hot water. There was no insoluble residue remaining on the filter from the casein digestion. There were rather large insoluble carbonaceous residues remaining from each of the casein-starch mixtures. Each of these was dried at 100° C., bottled, and set aside for further investigation as to their nitrogen content.

The filtrates in each of the experiments were concentrated separately under reduced pressure until practically all of the excess of hydrochloric acid was removed. The residues were taken up in water and run through filters into separate flasks of 250-c. c. capacity. After the filters were washed thoroughly, the contents of each flask were brought up to the mark with water, and duplicate analyses were carried out by the Van Slyke method on aliquots from each of these hydrolyzed solutions. The results obtained are shown in Table I.

From the data in Table I showing the average results obtained upon casein alone and upon definite mixtures of starch and casein digested at different intervals of time the following observations may be made.

In all of the experiments there is but slight variation in the ammonia determinations[1]; the maximum result is obtained in the 48-hour digestion. The increase in this case is in all probability owing to the change of some of the amino groups to ammonia compounds, which indicates over-digestion.

The results for the humin determinations show a diminution in the 15-, 24-, and 48-hour digestions over those of the 12-hour digestions. However, the humin determination in the 12-hour digestion of the starch-casein mixture agrees well with the humin results obtained on casein alone.

[1] Previous to the ammonia determinations the acidity of the hydrolyte, in terms of the calcium-hydrate suspension, was determined by titration, with phenolphthalein as the indicator. A slight excess of the calcium-hydrate suspension above the amount necessary to neutralize the acid was always added.

TABLE I.—*Effect produced upon the hydrolysis of casein in the presence of starch by varying the time of digestion*

| Amino-acid group. | Experiment 1 (10 gm. of casein+300 c. c. of 20 per cent hydrochloric acid; boiled for 12 hours). | | | Experiment 2 (10 gm. of casein+50 gm. of cornstarch+300 c. c. of 20 per cent hydrochloric acid; boiled for 12 hours). | | | Experiment 3 (10 gm. of casein+50 gm. of cornstarch+300 c. c. of 20 per cent hydrochloric acid; boiled for 15 hours). | | | Experiment 4 (10 gm. of casein+50 gm. of cornstarch+300 c. c. of 20 per cent hydrochloric acid; boiled for 24 hours). | | | Experiment 5 (10 gm. of casein+50 gm. of cornstarch+300 c. c. of 20 per cent hydrochloric acid; boiled for 48 hours). | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | No. 1 | No. 2. | Average. | No. 1. | No. 2. | Average. | No. 1. | No. 2. | Average. | No. 1. | No. 2. | Average. | No. 1. | No. 2. | Average. |
| | *Per cent.* | *Per cent.* | *Per cent.* | *Per cent.* | *Per cent.* | *Per cent.* | *Per cent.* | *Per cent.* | *Per cent.* | *Per cent.* | *Per cent.* | *Per cent.* | *Per cent.* | *Per cent.* | *Per cent.* |
| Ammonia nitrogen | 10.20 | 10.29 | 10.24 | 10.31 | 10.21 | 10.26 | 9.46 | 10.28 | 9.87 | 10.09 | 10.01 | 10.05 | 11.29 | 10.90 | 11.10 |
| Humin nitrogen | 1.32 | 1.32 | 1.32 | 1.47 | 1.47 | 1.47 | 1.10 | .75 | .92 | .95 | .95 | .95 | .74 | .80 | .77 |
| Arginin nitrogen | 7.99 | 7.90 | 7.94 | 6.49 | 7.06 | 6.77 | 5.46 | 6.21 | 5.83 | 6.32 | 6.23 | 6.27 | 6.00 | 6.00 | 6.00 |
| Histidin nitrogen | 7.94 | 8.15 | 8.04 | 8.18 | 8.18 | 8.18 | 6.93 | 5.90 | 6.41 | 1.19 | 1.79 | 1.49 | 2.71 | 2.59 | 2.65 |
| Cystin nitrogen | .14 | .17 | .15 | .19 | .16 | .17 | .15 | .19 | .17 | .07 | .06 | .07 | .07 | .05 | .06 |
| Lysin nitrogen | 9.13 | 9.50 | 9.31 | 8.79 | 8.82 | 8.80 | 8.03 | 9.47 | 8.75 | 10.37 | 9.85 | 10.11 | 11.58 | 9.88 | 10.73 |
| Amino nitrogen in filtrate from bases | 57.38 | 57.19 | 57.28 | 58.06 | 56.90 | 57.48 | 57.60 | 57.79 | 57.69 | 59.14 | 58.31 | 58.72 | 56.97 | 56.94 | 56.95 |
| Nonamino nitrogen in filtrate from bases | 8.67 | 8.26 | 8.46 | 8.47 | 10.31 | 9.36 | 11.15 | 8.93 | 10.04 | 8.80 | 10.17 | 9.48 | 8.37 | 9.69 | 9.03 |
| Total | 102.77 | 102.78 | 102.74 | 101.96 | 103.11 | 102.52 | 99.88 | 99.52 | 99.68 | 97.03 | 97.47 | 97.25 | 97.73 | 96.85 | 97.29 |

The results on humin represent the humin in solution and precipitated by calcium-hydrate suspension. The high results obtained by Grindley, Slater, et al. and Hart and Sure for humin nitrogen were made to include the total nitrogen in the insoluble residue and also the humin in solution.

It has been the experience of the writer that in cases where considerable insoluble residue was included in the total volume of the hydrolyte, great difficulty was met with in obtaining uniform aliquots for the total nitrogen in the solution and also for the aliquot for determination. This difficulty is avoided by filtering out and washing the insoluble residue. Then, too, the question arises, Is it fair to consider the nitrogen remaining in the insoluble residue as humin nitrogen?

The results for arginin show no serious loss in any of the determinations, and the minimum result obtained is only 1.5 per cent below Van Slyke's result for arginin on casein alone.

The histidin results are practically the same for the two 12-hour digestions, on casein alone and on the casein-starch mixture. In the 15-hour casein-starch digestion the result for histidin is 0.61 per cent above that reported in Van Slyke's analysis. In the 24- and 48-hour digestions there is a loss in histidin nitrogen of considerably more than 50 per cent of that found in the 15-hour digestion. Hence, the results for histidin in the two last experiments are very significant, indicating that long periods of digestion of starch and casein bring about a redistribution of the nitrogen in this group. It is to be borne in mind that Hart and Sure[1] obtained similar results on lysin. These writers also report 7.30 per cent as a average for histidin determinations in their experiment.

There is a diminution in the cystin nitrogen of more than 50 per cent in the 24- and 48-hour digestions. Hart and Sure state that their results for cystin were so low that they reported the results obtained by Van Slyke instead.

The results for lysin agree well in the 12- and 15-hour experiments. In the 24- and 48-hour experiments the results for lysin are high. Lysin nitrogen is obtained by deducting the sum of histidin, arginin, and cystin nitrogen from the total nitrogen in the bases; therefore any diminution in the nitrogen content of either histidin, arginin, or cystin will increase the results for lysin nitrogen correspondingly.

There is no marked difference between the results obtained in all the experiments for the amino- and nonamino-nitrogen content in the filtrates from the bases.

In the footings of the different analyses it is to be noted that the 12-hour digestions give footings more than 2.5 per cent over 100. In the 15-hour digestion the footing is good, while in the 24- and 48-hour digestions the footings are 2.75 per cent less than 100, thus indicating that the 12-hour experiments were probably not completely hydrolyzed;

[1] HART, E. B., and SURE, Barnett. Op. cit.

whereas the 15-hour digestion was sufficient to bring about complete hydrolysis and the 24- and 48-hour experiments were overdigested to the extent that nitrogen was lost.

The insoluble carbonaceous residues which were filtered from the hydrolyzed solutions were dried at 100° C. and the total nitrogen determined in each.

The insoluble residue from experiment 2, or the 12-hour starch-casein digestion, contained 1.30 per cent of nitrogen. That from the 15-hour digestion contained 0.83 per cent of nitrogen. That from the 24-hour digestion contained 0.80 per cent of nitrogen and that from the 48-hour digestion contained 0.855 per cent of nitrogen. The results show that a 15-hour digestion removed as much nitrogen from the insoluble residue as the 24- and 48-hour digestions.

Two determinations of total nitrogen on a sample of the dry starch showed an average nitrogen content of 0.05 per cent. The small amount of nitrogen contained in the starch and the comparatively greater amount found in the insoluble residues indicate that some nitrogen compound was absorbed by the latter.

Seven gm. of the dry-carbon residue were weighed out and transferred to a Claisen flask, 60 c. c. of a 10 per cent calcium-hydrate suspension added, together with 250 c. c. of distilled water. The apparatus was connected up as in an ammonia determination and distilled under reduced pressure at from 40° to 45° C. for 30 minutes. Nine-tenths c. c. of *N/10* hydrochloric acid was neutralized by the ammonia evolved, which shows that the insoluble-carbon residue contained only a trace of ammonia nitrogen. The insoluble-carbon and calcium-hydrate precipitate remaining in the Claisen flask was filtered and washed thoroughly, the filtrate made acid and concentrated under reduced pressure to about 50 c. c. The concentrate was transferred to a Kjeldahl flask and the total nitrogen determined in the usual way. The filtrate contained 0.0032 gm. of nitrogen or 5.3 per cent of the total nitrogen contained in the insoluble residue. The ammonia nitrogen was 2.1 per cent of the total nitrogen in the carbon residue. It is therefore evident that a very small percentage of the total nitrogen contained in the insoluble residue is affected by distilling with calcium-hydrate suspension, which indicates that the nitrogen remaining in the insoluble-carbon residue after digestion and washing is in what may be considered an inert form and should not be included in the humin group.

## CONCLUSIONS

From the data contained in this paper the following conclusions may be drawn:

(1) The Van Slyke method for protein analysis, when applied to mixtures of casein and starch in the proportion of 1 to 5, and hydrolyzed

from 12 to 15 hours with 20 per cent hydrochloric acid gives results for the amino-acid groups that are comparable with those obtained by Van Slyke upon casein alone.

(2) A digestion period of more than 15 hours with 20 per cent hydrochloric acid on a casein-starch mixture brings about a redistribution of the nitrogen contained in the histidin and cystin groups.

(3) The insoluble residue obtained from a casein-starch digestion after being thoroughly washed contains nitrogen, which is not seriously affected when distilled with calcium-hydrate suspension, very small amounts being split off as ammonia or remaining in the filtrate. This indicates that the nitrogen is in an inert form and its estimation should not be included in the humin determination.