[CONTRIBUTION FROM THE DIVISION OF AGRICULTURAL BIOCHEMISTRY, MINNESOTA AGRICULTURAL EXPERIMENT STATION]

PROLINE AND TRYPTOPHAN AS FACTORS INFLUENCING THE ACCURACY OF VAN SLYKE'S METHOD FOR THE DETERMINATION OF NITROGEN DISTRIBUTION IN PROTEINS[1]

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Van Slyke[2] in 1911 described a procedure for the differentiation of proteins by the determination of certain chemical groups characteristic of certain of the amino acids. His method was essentially a combination of the earlier Hausmann[3] method as modified by Osborne and Harris[4] with his own method for the determination of amino nitrogen groups.[5] This method has been extensively used by protein investigators in recent years. In his original study of the method, Van Slyke prepared a known mixture of amino acids and subjected this mixture to the proposed scheme of analysis, securing very accurate results. Van Slyke[5] states that "the test of the method would be made more complete by analysis of a mixture like the above but containing tryptophan and boiled 24 hours with 20% hydrochloric acid. Unfortunately, the completion of this experiment was prevented by the expiration of the period available for the work, which cannot be taken up again for a number of months."

In so far as we are aware, this suggested experiment has never been carried out. Van Slyke in a private communication states that he has not been able to carry out the work due to pressure of work along other lines.

The question as to the effect of acid hydrolysis and of the presence of tryptophan upon the accuracy of Van Slyke's method can be ascertained only by actual experiments using mixtures of pure amino acids as the material under investigation. Henriques and Gjaldbäk,[6] Van Slyke,[7] and later Gortner and Holm[8] have shown that when proteins are hydrolyzed with 20% hydrochloric acid some deamination takes place with a resulting augmentation of the ammonia fraction. Morner,[9] Van Slyke,[2] and Hoffman and Gortner[10] have shown that the properties of cystine may be profoundly altered by boiling with strong acids. Tryptophan is a component of a majority of the proteins,

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[2] Van Slyke, *J. Biol. Chem.*, **10**, 15 (1911).

[3] Hausmann, *Z. physiol. Chem.*, **29**, 136 (1900).

[4] Osborne and Harris, THIS JOURNAL, **25**, 323 (1903).

[5] Van Slyke, *J. Biol. Chem.*, **9**, 185 (1911); **12**, 275 (1912).

[6] Henriques and Gjaldbäk, *Z. physiol. Chem.*, **67**, 8 (1910).

[7] Van Slyke, *J. Biol. Chem.*, **12**, 295 (1912).

[8] Gortner and Holm, THIS JOURNAL, **39**, 2736 (1917).

[9] Morner, *Z. physiol. Chem.*, **34**, 207 (1901–2).

[10] Hoffman and Gortner, THIS JOURNAL, **44**, 341 (1922).

and appears to be an extremely labile compound under conditions such as occur in a protein hydrolysis. Van Slyke[2] has shown that tryptophan is altered or decomposed under the conditions prevailing in a protein hydrolysis and Holm and Gortner[11] have shown that tryptophan under such conditions distributes itself in all of the Hausmann fractions.

Early studies from this Laboratory[12] have demonstrated that the nitrogen in the humin of protein hydrolysis has its origin in the tryptophan molecule but that the tryptophan is not quantitatively removed in the humin fraction unless some second component of the protein molecule (probably an aldehyde) is present in exactly equivalent quantity. It appears doubtful whether this condition is ever realized in a natural protein. In many cases the aldehydic component is present in too small an amount to combine with all of the tryptophan, with the result that humin formation does not reach a maximum nor does the humin nitrogen bear any direct relation to the tryptophan content of the protein. This is certainly the case in casein and fibrin. The result of such a condition is that a portion of the tryptophan nitrogen is estimated as humin nitrogen and the balance is distributed in the same manner as if no humin formation had taken place.

It accordingly seemed desirable to determine the nitrogen distribution in a mixture of known amino acids, using Van Slyke's method of protein analysis but to vary the conditions, so as to ascertain what influence the boiling with acid or the presence of tryptophan would have upon the final values. As a secondary problem we have also included the presence and absence of proline as a factor in the accuracy of the method, inasmuch as it is well known that proline forms a relatively insoluble phosphotungstate.[13]

Experimental Part

Three artificial mixtures of amino acids were used in the experiments. Table I shows the amino acids, their sources, the quantities used and their distribution as amino and non-amino nitrogen. All of the amino acids used were weighed out, dissolved and made to volume in a calibrated flask. From this, aliquot portions for the different experiments were pipetted. The tryptophan was weighed and added to the proper mixtures. It was impossible to obtain any proline entirely free from amino nitrogen, and therefore our purest preparation was dissolved in water and analyzed for both amino nitrogen and total nitrogen. This proline was prepared from gelatin by Dakin's butyl alcohol method,[14] followed by precipitation with phosphotungstic acid and the decomposition of the phosphotungstate with barium hydroxide, and subsequently by conversion twice through the copper salt procedure retaining only the alcohol-soluble copper salt, and finally repeated solution of the free amino acid in absolute alcohol and precipitation with ether. An analysis of the solution of this material

[11] Holm and Gortner, THIS JOURNAL, **42**, 2378 (1920).

[12] *Ibid.*, **37**, 1630 (1915); **39**, 2477 (1917); **42**, 632 (1920); **42**, 821 (1920); **42**, 2378 (1920); **45**, 550 (1923); **46**, 1224 (1924); *J. Biol. Chem.*, **26**, 177 (1916).

[13] Sörensen, *Compt. rend. trav. lab. Carlsberg*, **6**, 168 (1905).

[14] Dakin, *Biochem. J.*, **12**, 290 (1918).

showed that a 1cc. aliquot portion contained 0.539 mg. of total nitrogen, of which 0.052 mg. was amino nitrogen as liberated in 30 minutes in the Van Slyke apparatus. The literature contains no definite statement as to the behavior of proline in the Van Slyke apparatus. Numerous attempts have been made in this Laboratory to prepare proline that did not liberate nitrogen in the apparatus, but in no instance has such an attempt been successful. It is possible that the pyrrolidine ring opens to some extent in the treatment with nitrous acid. The arginine used was prepared from the crystallized picrate by adding hydrochloric acid and shaking out the picric acid with ether until the solution was colorless. Total nitrogen and amino nitrogen were then determined on the solution; 1 cc. contained 1.987 mg. of total nitrogen and 0.4827 mg. of amino nitrogen.

TABLE I

AMINO ACIDS USED, SOURCES, QUANTITIES TAKEN AND THE WEIGHTS OF AMINO AND OF NON-AMINO NITROGEN PRESENT

Amino acid	Source	Amount used in each experiment G.	Total nitrogen, mg.	Amino nitrogen, mg.	Non-amino nitrogen, mg.
Alanine	Silk	0.1500	23.58	23.58	...
Arginine hydrochloride	Edestin	.1983	63.80	15.95	47.85
Aspartic acid	Kahlbaum	.1500	15.80	15.80	...
Cystine	Human hair	.1250	14.56	14.56	...
Glutamic acid hydrochloride	Gelatin and casein	.2000	15.20	15.20	...
Glycine	Silk	.2000	37.34	37.34	...
Histidine dihydrochloride	Blood	.2500	46.05	15.35	30.70
Leucine	Gelatin and casein	.1500	16.04	16.04	...
Lysine dihydrochloride	Gelatin and casein	.1000	12.79	12.79	...
Norleucine	Synthetic	.1500	16.04	16.04	...
Phenylalanine	Gelatin and casein	.1500	12.74	12.74	...
Proline[b]	Gelatin	.1500	18.26	1.76	16.50
Serine	Synthetic	.1250	16.68	16.68	...
Tryptophan[c]	Casein	.1000	13.72	6.86	6.86
Tyrosine	Casein	.1500	11.61	11.61	...
Valine	[a]	.1250	14.95	14.95	...

[a] We are indebted to Dr. C. O. Johns for this sample of valine.
[b] In Expt. 3 only.
[c] In Expt. 2.

Each experiment was made in two parts; one half of the material was analyzed exactly according to the Van Slyke procedure except that it was never boiled. The second portion was gently boiled for 24 hours with 75 cc. of constant-boiling hydrochloric acid on a sand-bath. The data are corrected for the solubility of the basic phosphotungstates as recommended by Van Slyke.

Color Changes.—All the unboiled solutions were clear, with just a faint straw color. After hydrolysis, the liquids in Expts. 1 and 3 were not appreciably changed in color, although they had darkened slightly; that of Expt. 2 was a dark red to brown and was almost opaque although no solid particles could be seen. In no case did a solution leave a colored

residue on the filter (the acid-insoluble humin). This was to be expected, providing aldehydes were absent.

In Expt. 2, containing tryptophan, much of the color was removed by the calcium hydroxide precipitate, although the solution was still of a light mahogany color. The phosphotungstic acid precipitate always contained some color, being a pale dirty yellow, and the filtrate showed only a pale straw-yellow color, much like the original solutions.

Analytical Data.—The results of the three experiments are given in Tables II, III and IV. All data are expressed as percentages of the total

TABLE II

OBTAINED IN EXPERIMENT 1 (PROLINE AND TRYPTOPHAN ABSENT) AND DEVIATIONS FROM CALCULATED VALUES

	Analyses			Deviations		
	A Calculated %	B Unboiled %	C Boiled %	B−A %	C−B %	C−A %
Ammonia	...	0.00	0.87	0.00	+0.87	+0.87
Soluble humin	...	.02	.14	+ .02	+ .12	+ .14
Bases—total nitrogen	43.10	43.31	40.80	+ .21	−2.51	−2.31
amino nitrogen	18.47	18.42	16.54	− .05	−1.88	−1.93
non-amino nitrogen	24.63	24.89	24.26	+ .26	−0.63	−0.37
Filtrate—total nitrogen	56.93	54.96	55.63	−1.97	+ .67	−1.30
amino nitrogen	56.93	54.55	54.61	−2.38	+ .06	−2.32
non-amino nitrogen	...	0.41	1.02	+0.41	+ .61	+1.02
Phosphotungstic acid humin	...	.86	0.97	+ .86	+ .11	+0.97
Cystine	4.60	4.59	2.97	− .01	−1.62	−1.63
Arginine	19.89	19.88	19.84	− .01	−0.04	−0.05
Histidine	14.56	14.97	14.07	+ .41	− .90	− .49
Lysine	4.05	3.87	3.92	− .18	+ .05	− .13
Recovery		99.14	98.40			

TABLE III

DATA OBTAINED IN EXPERIMENT 2 (PROLINE ABSENT, TRYPTOPHAN PRESENT) AND DEVIATIONS FROM CALCULATED VALUES

	Analyses			Deviations		
	A %	B %	C %	B−A %	C−B %	C−A %
Ammonia	...	0.03	0.64	+0.03	+0.61	+0.64
Soluble humin	...	.00	.94	.00	+ .94	+0.94
Bases—total nitrogen	41.31	42.91	44.05	+1.60	+1.14	+2.74
amino nitrogen	17.71	18.26	17.53	+0.55	−0.73	−0.18
non-amino nitrogen	23.60	24.65	26.52	+1.05	+1.87	+2.92
Filtrate—total nitrogen	58.70	54.02	49.92	−4.68	−4.10	−8.78
amino nitrogen	56.62	51.58	47.68	−5.04	−3.90	−8.94
non-amino nitrogen	2.08	2.44	2.24	+0.36	−0.20	+0.16
Phosphotungstic acid humin	...	0.94	1.29	+ .94	+ .35	+1.29
Cystine	4.41	4.57	2.74	+ .16	−1.83	−1.67
Arginine	19.06	20.34	20.56	+1.28	+0.22	+1.50
Histidine	13.96	14.10	16.75	+0.14	+2.55	+2.69
Lysine	3.88	3.90	4.00	+ .02	+0.10	+0.12
Recovery		99.40	97.78			

TABLE IV

DATA OBTAINED IN EXPERIMENT 3 (PROLINE PRESENT, TRYPTOPHAN ABSENT) AND DEVIATIONS FROM CALCULATED VALUES

	Analyses			Deviations		
	A %	B %	C %	B−A %	C−B %	C−A %
Ammonia	...	0.02	0.87	+0.02	+0.85	+0.87
Soluble humin	...	.02	.07	+ .02	+ .05	+ .07
Bases—total nitrogen	40.75	45.30	44.51	+4.55	− .79	+3.76
amino nitrogen	17.46	18.18	18.09	+0.72	− .09	+0.63
non-amino nitrogen	23.29	27.12	26.42	+3.83	− .70	+3.13
Filtrate—total nitrogen	59.25	52.65	51.31	−6.60	−1.34	−7.94
amino nitrogen	53.76	48.06	44.56	−5.73	−3.50	−9.23
non-amino nitrogen	5.46	4.59	6.75	−0.87	+2.16	+1.29
Phosphotungstic acid humin	...	1.22	1.25	+1.22	+0.03	+1.25
Cystine	4.35	4.70	3.19	+0.35	−1.51	−1.16
Arginine	18.80	20.75	20.90	+1.95	+0.15	+2.10
Histidine	13.77	17.34	16.12	+3.57	−1.22	+2.35
Lysine	3.82	2.51	4.31	−1.31	+1.80	+0.49
Recovery		99.17	98.00			

nitrogen. For each experiment there are three headings: (A)—"Calculated," the values calculated on the assumption that all the hexone bases remain in the phosphotungstic acid precipitate, and the other amino acids pass into the filtrate from the bases; (B)—"Unboiled," the result of analyzing the mixture after having added the hydrochloric acid, but without boiling it for 24 hours; (C)—"Boiled" for 24 hours, the analysis, as under B, except that the mixture was boiled for 24 hours with 20% hydrochloric acid.

Discussion

The errors in the Van Slyke method of protein analysis have been calculated and are shown in the last three columns of Tables II, III and IV.

"B − A" represents the difference between the calculated value and the analysis of the unboiled mixture of amino acids; it gives the error due to manipulation plus that inherent in the method.

"C − B" shows the difference between analyses of the boiled and unboiled aliquots. This represents the changes produced by boiling the amino acids for 24 hours with 20% hydrochloric acid.

"C − A" represents the difference between the values for the boiled aliquot portions and the calculated values. This gives the net errors in the method.

A comparison of differences between calculated values and the actual analysis of the unboiled mixture in Expt. 1 and Expt. 3 with the results given by Van Slyke[2] is of interest and is shown in Table V. The latter's figures were given in grams, which we have calculated to percentages.

The material used in our Expt. 3 containing proline, is comparable to Van Slyke's mixture. It is to be noted that he omitted tryptophan from

his artificial hydrolysate, and consequently our material is not directly comparable with his mixture.

TABLE V

EXPERIMENTAL ERRORS OF ANALYSIS IN AN ARTIFICIAL MIXTURE OF AMINO ACIDS

	Van Slyke's differences G.	Van Slyke's differences %	B − A, Expt. 1	B − A, Expt. 3
Total N	0.4289	.....	....	....
NH_3	+ .0008	+0.186	....	+0.02
Arginine	+ .0006	+ .139	−0.01	+1.95
Cystine	− .0001	− .023	− .01	+0.35
Histidine	− .0037	+ .862	+ .41	+3.57
Lysine	+ .0014	− .326	− .18	−1.31
NH_2 filtrate	+ .0049	+1.141	−2.38	−5.73
Non-NH_2 filtrate	− .0024	−0.558	+0.41	−0.87
	+ .0015	+ .349	−1.76	−2.02

We found more of a loss of the amino-nitrogen fraction in the filtrate from the bases than did Van Slyke. In Expt. 1, where both tryptophan and proline are absent, the results agree fairly well with those reported by Van Slyke. However, our values for the hexone bases change somewhat when proline is present, indicating that this amino acid is partially precipitated by phosphotungstic acid, since proline theoretically contains no amino nitrogen. Since proline was present to the extent of 5.45%, we find a gain of 0.7% of total nitrogen, or 12.8% of proline nitrogen, coming down in the phosphotungstic acid precipitate. This would tend to increase the histidine fraction which agrees with our findings. An increase in histidine value with no similar increase in total nitrogen of the bases will lower the value of the lysine found.

When we examine our findings as shown in B − A of Expt. 2, the results are not so striking. The gain of 1.28% for arginine must be an analytical error, since it can be determined more accurately than this. Consequently, although both the amino nitrogen and the non-amino nitrogen of the phosphotungstic acid precipitate were too high, the histidine value does not reflect this. However, the increases of both amino and non-amino nitrogen in the phosphotungstic acid precipitate must be due to the tryptophan, which hypothesis is borne out by the fact that the nitrogen in the filtrate is correspondingly low. This is also observed in the case of Expt. 3 where proline was used.

The major question, however, is: what changes take place when the amino acids are boiled for 24 hours with 20% hydrochloric acid? Such changes are given in the tables in the columns headed C − B.

The Ammonia Fraction.—The boiling produces some ammonia (0.61–0.87%). This agrees with much of the earlier work where deamination was found to take place whenever a protein was boiled for longer than a few hours.[15]

[15] *Z. physiol. Chem.*, **67**, 8 (1910). THIS JOURNAL, **39**, 2736 (1917); **42**, 821 (1920); **44**, 341 (1922).

Insoluble Humin.—In this study we found no insoluble humin, although it is to be noted that when the mixture of amino acids containing tryptophan was boiled, a marked darkening appeared. This is in agreement with earlier findings.[12] It was noted that much of this color was removed in the precipitation with lime, and some with the barium phosphotungstate.

Soluble Humin.—It is interesting to note that the acid-soluble fraction was low in the experiments where no tryptophan was used (0.12 and 0.05% resulted from boiling). However, in Expt. 2 where tryptophan also was present, the soluble humin fraction becomes appreciable (0.94%).

The Basic Nitrogen.—By comparing Expts. 1, 2 and 3, we note an appreciable increase in the total basic nitrogen in the last two experiments where tryptophan and proline are present. In Expt. 2 where tryptophan is used, we find an increase in both the amino (+0.55%) and the non-amino (+1.05%) portions. These are not equal as theoretically required, but support is lent to the theory that they arise from the tryptophan when we note that the filtrate from the bases shows deficiencies in these fractions. Van Slyke pointed out that tryptophan might precipitate in this fraction. When the mixture is boiled, less tryptophan is precipitated by phosphotungstic acid. This is due to the fact that the tryptophan is altered as has been shown by Gortner and Holm,[8] who found that 15% of deamination had occurred during the boiling for 24 hours.

In Expt. 3 where proline was added, it likewise appeared in the phosphotungstate precipitate. The unboiled solution gained 3.76% in total nitrogen of which 3.13% was non-amino, while the filtrate lost in total nitrogen to the extent of 7.94%; of this 4.59% was non-amino nitrogen. After boiling, much the same condition obtains, for the bases gained 4.55% in total nitrogen, while the filtrate lost 6.60% in total nitrogen. Of this, the bases gained 3.13% in non-amino nitrogen while the filtrate lost 0.87%. The discrepancies in these figures may be due partly to experimental error and partly to the fact that the filtrate from the bases appears to gain some non-amino nitrogen on boiling, possibly due to anhydride formation.

Arginine.—Arginine was recovered within experimental error. The figures are a little higher than the theoretical even when corrected by deducting 18% of the cystine nitrogen. The increase is so slight that it is a question as to its interpretation. In this connection it is of interest to recall Plimmer's findings[16] that somewhat less than 3% (1.5–3%) of the histidine nitrogen appeared as ammonia liberated in the arginine determination.

Cystine.—The cystine values are interesting, and confirm the earlier studies. The amounts that we recovered after boiling were 64.5, 62.1 and 73.3% in the three experiments.

[16] Plimmer, *Biochem. J.*, **10**, 115 (1916).

Histidine.—In Expt. 1 (no proline or tryptophan) the histidine was satisfactorily determined (14.07 and 14.97% as compared with the calculated 14.56%). However, in the presence of tryptophan or proline, too high results were obtained. In Expt. 2 (tryptophan present), 16.75% (unboiled) and 14.10% (boiled) were obtained as compared with a calculated value of 13.96%; and in Expt. 3 (proline present), 16.12% (unboiled) and 17.34% (boiled) from a 13.77% fraction. The figures indicate that tryptophan and proline are precipitated in the bases of Expts. 2 and 3 to the extent of 11.6 and 24.9%, respectively, which fact accounts for the high values for histidine.

Lysine.—The lysine fraction is determined by difference and is not so striking. This is due to the fact that the histidine fraction absorbs most of the excess of total nitrogen as well, since the error in the non-amino nitrogen will also involve the use of some of the amino nitrogen, on the basis that one third of the histidine nitrogen is amino nitrogen. Thus 4.00 and 3.90% of lysine nitrogen were recovered as compared with the calculated value of 3.88%, and 4.31 and 2.51% as compared with the calculated value of 3.82%.

Phosphotungstic Acid Humin.—Our experiments do not throw any light on the origin of this fraction. A study of the data does not show any regularity in the variations between the boiled and the unboiled mixtures.

Summary

Van Slyke's method for the determination of the distribution of nitrogen in proteins has been tested on a mixture of amino acids before and after boiling for 24 hours in the presence of hydrochloric acid, both in the presence and in the absence of proline or tryptophan. The data point to the following conclusions.

1. In the absence of tryptophan and of proline and without previous boiling of the amino acids, the method yields essentially correct results, thus confirming the original work of Van Slyke.

2. When a mixture of 14 amino acids (not including tryptophan or proline) is boiled for 24 hours prior to analysis, the resulting analysis shows that approximately 35.5% of the cystine nitrogen is not precipitated by phosphotungstic acid, causing a corresponding loss in the amino nitrogen of the bases. There is a gain in the ammonia nitrogen. These changes are the only ones appreciably greater than the experimental errors of the method.

3. When tryptophan is added to the mixture of 14 amino acids (no proline) noted above, the analysis of the unboiled sample showed appreciable errors in the basic fraction and in the amino nitrogen and the total nitrogen of the filtrate from the bases. The errors in the basic nitrogen affect chiefly the arginine fraction.

4. The analysis of the boiled mixture with tryptophan present shows errors in the histidine and the cystine fractions of the bases (cystine 37.9% not precipitated), in the ammonia fractions and in the filtrate from the bases.

5. When proline is added to the mixture of 14 amino acids (no tryptophan present) and the unboiled mixture is then analyzed, errors are found both in the basic fraction and in the fractions in the filtrate from the bases. Apparently, phosphotungstic acid precipitates a part of the proline with the diamino acids. This proline nitrogen distributes itself between the arginine and the histidine fractions, and because of its entire lack of amino nitrogen, the calculations of Van Slyke's method cause the lysine fraction to show a loss.

6. When the mixture containing proline is boiled for 24 hours prior to analysis the basic nitrogen fractions still far exceed the calculated values, with a corresponding decrease in the fractions of the filtrate. Cystine again is only partially precipitated (73.3%) and the ammonia nitrogen is increased.

7. In general, the data show that both tryptophan and proline produce errors in the Van Slyke nitrogen distribution if they are present in a protein or a mixture of amino acids.

8. The cystine value of a Van Slyke analysis on a 24-hour protein hydrolysate may be taken to represent approximately 65% of the true cystine nitrogen present in the unboiled material.

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THE EFFECT OF CALCIUM SULFATE ON THE GROWTH AND FERMENTATION OF YEAST[1]

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While various observers have determined the effect of the chloride and the carbonate of calcium on the growth and fermentation of yeast, no one has studied the effect of the sulfate. Since the sulfate is the "permanent hardness" of water, it seemed desirable to study the effect of this salt on a pure culture[2] of *Saccharomyces cerevisiae* under controlled conditions.

The culture medium was the same as that used by Williams[3] except that the calcium chloride was omitted. The medium was made up in a concentrated solution

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[2] Isolated for the writer through the courtesy of Mr. H. E. Turley of the American Bakers Association.

[3] Williams, *J. Biol. Chem.*, **42**, 260 (1920).