CVIII. THE TITRATION OF PROTEIN HYDROLYSATES.

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(Received June 15th, 1927.)

FOREMAN [1920] and HARRIS [1924] have shown that the acid radicles of amino-acids can be titrated quantitatively if the K_a of such amino-acids in solution were increased sufficiently to afford a sharp reaction with the indicators used. This was effected by titrating in strong alcoholic solution (above 87 % alcohol) to phenolphthalein in Foreman's method and to thymolphthalein in Harris's method. Better results were obtained by the addition of formaldehyde to the alcoholic solution according to Sørensen's method [1907]. Also, by the inclusion of formaldehyde in the solutions, difficulties in obtaining accurate results with dicarboxylic acids and diamino-acids were overcome.

The procedure was to neutralise the *aqueous* solutions to the respective indicators and, after adding the requisite volume of neutralised solvent to the solution, again to neutralise to the indicator used. This increase in acidity was the basis for calculating the acid radicle present in the solutions. Work was done on most of the common amino-acids individually so that data of the behaviour of all the different types of amino-acids under such conditions have been collected.

Attention has been paid in the present work to the actual distribution of nitrogen in solutions containing mixtures of amino-acids and not to the total acid radicle present. The principle of weight of nitrogen equivalent to titratable acid radicle has, therefore, been considered. With a solution of glycine hydrochloride, the hydrochloride portion would be neutralised completely in aqueous solution with alkali to phenolphthalein, whereas in the subsequent alcoholic titration, the carboxyl group would be quantitatively neutralised. The titre of the carboxyl group would be exactly equivalent to the weight of nitrogen present in the glycine solution, *i.e.* every 1 cc. of normal alkali used in the alcoholic titration would be equivalent to 0.014 g. of nitrogen.

From Foreman's paper [1920] the following deductions have been drawn concerning the nitrogen of the various amino-acids equivalent to the titratable value of such acids in alcohol-formalin solution after previous neutralisation in aqueous solution:

Monoamino-acids.	All the nitrogen.
Dibasic acids.	All the nitrogen (one carboxyl group is neutralised in aqueous solution).
Cystine and lysine.	All the nitrogen.
Arginine.	One-quarter of the nitrogen.
Histidine.	One-third of the nitrogen.

Ammonium salts enter into the same class as monoamino-acids.

With the above data, the alcohol titration value of a mixture of amino-acids in solution could be calculated, even if the nitrogen distribution (Van Slyke) only were known (provided, of course, that the various amino-acids did not react together in alcohol-formalin solution, thus upsetting the actual titration values of the amino-acids collectively); *e.g.* if the distribution of nitrogen in the diamino-acids plus cystine fraction in the Van Slyke method were correct for each amino-acid, the titration value of that solution could be calculated.

WORK ON PROTEIN HYDROLYSATES.

A. *Titration values of complete hydrolysates compared with the sum of the titration values of the fractions separated by the Van Slyke method.*

Gelatin. 18 g. of pure gelatin were hydrolysed with 20 % hydrochloric acid for 36 hours and the hydrolysate evaporated twice to dryness *in vacuo*. The gummy residue was dissolved in water and decolorised with a small amount of Merck's medicinal charcoal and made up to a litre. 150 cc. were proceeded with in the ordinary VanSlyke method of separating into groups. The phosphotungstates of the diamino-acids plus cystine were decomposed by the ether-amyl alcohol method, the solution of amino-acids being freed as much as possible from hydrochloric acid and made up to 100 cc. Excess of phosphotungstic acid was removed from the filtrate by ether-amyl alcohol and the solution freed from as much hydrochloric acid as possible and made up to 250 cc. Alcohol-titration values of the original solution, filtrate, diamino-acid plus cystine fraction, and the equivalent of the amide nitrogen were determined.

Caseinogen (commercial sample 1). The same procedure as for gelatin was carried out for 18 g. of a sample of commercial caseinogen. The alcohol titration values of the original solution, the filtrate, the diamino-acid plus cystine fraction, and the equivalent of the amide nitrogen were determined.

Table I. *Gelatin and caseinogen hydrolysates. Comparison of the alcohol-titration values of the complete hydrolysates against those of the fractions separated by the Van Slyke method.*

	Gelatin	Caseinogen
Total nitrogen (g.)... ..	0.4644	0.4245
Titration value (cc. 0.1 N alkali) of complete hydrolysate	271.8	253.9
Filtrate	218.8	172.0
Diamino-acids plus cystine	44.7	52.3
Equivalent of amide-N	7.5	30.4
Total of fractions	271.0	254.7
Titration equivalent as g. nitrogen	0.3794	0.3566
Nitrogen not titrated (g.)... ..	0.0850	0.0679
„ „ (% total nitrogen)	18.30	16.00

From Table I it can be seen that the sums of the titration values of the fractions were almost identical with the titration values of the original solutions. Hence, there has been no change in the titration values of the hydrolysates effected by the process of separating the fractions by the Van Slyke method.

B. The titration values of the diamino-acid plus cystine fractions of various proteins.

Three separate samples of caseinogen, I and II being commercial samples and III a pure sample, were investigated, each in duplicate, for distribution of nitrogen according to the Van Slyke method. The gelatin cited in A above was also studied in the same direction in duplicate (150 cc. per determination). During the course of the author's work [Davies, 1927] on the proteins of green forage plants, eight samples of proteins were prepared from green brassicaceous plants and two from umbelliferous plants and analysed by the Van Slyke method in duplicate. For each of the above proteins the different amounts of nitrogen allotted to arginine, histidine, lysine and cystine were worked out in the usual way. Part of the diamino-acid solution in each determination was used to determine the titration value in alcohol-formalin solution (after previous neutralisation of the aqueous solution to phenolphthalein).

From the amounts of nitrogen allotted in each case to the different amino-acids (not corrected for the solubilities of the bases) the theoretical titration value was calculated as cc. 0.1 *N* alkali (*i.e.* one-fourth arginine-N, one-third histidine-N, total lysine- and cystine-N $\div$.0014). The results obtained are given in Table II.

Table II. *Calculated and actual alcohol-titration values of the diamino-acids (Van Slyke method) of various proteins.*

Source of protein	N per determination (g.)	Alcohol-titration values of diamino-acid fraction as cc. 0.1 <i>N</i> alkali		Calculated: found
		Calculated	Found	
Caseinogen I	0.4245	41.36	52.30	1.27
Caseinogen II	0.4511	35.79	44.10	1.23
Caseinogen III	0.4674	47.64	57.90	1.22
Gelatin	0.4644	36.36	44.74	1.23
Cabbage I	0.4372	42.00	51.35	1.25
Kohlrabi	0.4263	41.65	49.78	1.20
Marrow stem kale leaves	0.4089	41.71	50.34	1.21
Ditto. Stems	0.4576	42.71	54.24	1.27
Turnip leaves	0.5355	53.10	63.08	1.19
Turnip root	0.4750	51.07	60.52	1.19
Cabbage II	0.4167	35.57	43.63	1.23
Turnip leaves II	0.3906	31.79	36.84	1.16
Carrot	0.3814	30.93	39.30	1.23
Parsnip	0.4286	42.15	50.00	1.19

Average = 1.22

(The Van Slyke distributions of nitrogen were determined in duplicate for each of the above proteins, the average values only being used in compiling Table II.)

EXPERIMENTAL NOTE. *N*/10 aqueous alkali was used for the aqueous neutralisation and *N*/25 alcoholic potash for the alcoholic titration. 10 cc. (*ex* 100 cc. of diamino-acid solution) required from 9 to 12 cc. alcoholic potash for the titration.

C. Titration of the monoamino-acid fraction (the filtrate of the Van Slyke method).

The monoamino-acid fractions for most of the proteins of Table II had been treated with caustic soda and hydrochloric acid and were, therefore, unsuitable for amino-acid titration owing to separation of salts from alcoholic solution. Samples of filtrate from gelatin and caseinogen I were treated with ether-amyl alcohol to get rid of the excess phosphotungstic acid used to precipitate the bases. The solutions were evaporated to dryness twice and made up to a known volume, the titration values of aliquot portions of these being then determined (*v.* Table III).

Table III. *Titration of the specially treated monoamino-acid fractions of protein hydrolysates (obtained by the Van Slyke method).*

Protein	Total nitrogen in hydrolysate (g.)	Nitrogen in filtrate (g.)	Titration value cc. N/10 alkali		Calculated: found
			Calculated	Found	
Gelatin	0.4245	0.3328	237.7	226.3	0.95
Caseinogen I	0.4644	0.2608	196.3	172.0	0.92

EXPERIMENTAL NOTE. N/10 alcoholic potash was the strength of alkali best suited for the titration. 10 cc. (*ex* 250 cc. total solution of monamino-acids) requiring 6 to 7 cc. N/10 alkali for the alcoholic titration.

SIGNIFICANCE OF THE VALUES DETERMINED BY ALCOHOLIC TITRATION.

From Table I it is apparent that there has been no change in the titration values of the hydrolysates due to the processes of separating the fractions, and that losses during the operations were negligible. From Table II the titration values found for the diamino-acid solutions were uniformly greater than the expected values as calculated from the figures obtained by the Van Slyke method by 22 % of the latter.

Table III shows that the increase in titration apparent in Table II is balanced by the filtrate showing a lower titration value than that calculable for a monoamino-acid solution (a decrease of 8 % on the average). Theoretically, this solution should contain no nitrogen not accounted for by titration. As it is, the gelatin filtrate contained 0.016 g. and the caseinogen 0.020 g. not accounted for in the titration. These nitrogens, undoubtedly, belong to the bases, arginine and histidine, being the amounts not precipitated by phosphotungstic acid. Plimmer and Rosedale [1925] have advised the determination of arginine in the original hydrolysate, since its determination in the phosphotungstic precipitate gives low values. This fact is supported in this work. Van Slyke [1915] corrects for the solubility of the bases by adding 0.0032 g. nitrogen to the arginine nitrogen and 0.0038 g. to the histidine nitrogen, the total corresponding to 0.0050 g. of *untitratable* nitrogen in the filtrate. From the present work it is obvious that these corrections are too low. Plimmer and Rosedale have made similar deductions.

THE RELATION OF THE TITRATION VALUES TO THE AMINO-NITROGEN.

For the diamino-acids the titration values should be equivalent to the amino-nitrogen. That is, the equivalent of the sum of the lysine and cystine nitrogen, a third of the histidine nitrogen and a quarter of the arginine nitrogen should equal the titration value of the bases in alcoholic solution. Table II, columns 2 and 3, shows the calculated and observed values for the series of proteins. The equivalent found by titration is uniformly 22 % higher than the calculated value. Plimmer [1924] has observed the unsatisfactory results obtained by the method prescribed by Van Slyke [1915] for the determination of the amino-nitrogen of the bases. The specified time for reaction with nitrous acid is not sufficient for all the amino-nitrogen, especially that of lysine, to react and Plimmer has introduced longer periods than 5 minutes' shaking so as to get more accurate results. The results for the series of proteins (Table II) were obtained by the original Van Slyke method, and this discrepancy between calculated and observed values bears out Plimmer's observations. The uniformity of the discrepancy also seems to point out that, irrespective of the composition of the phosphotungstic precipitate, only about 82 % of the amino-nitrogen reacts during the 5 minutes' shaking. (The actual discrepancy cannot, of course, be calculated since the only basis of calculation is afforded by incorrect results.)

It is tentatively suggested, therefore, that alcohol-titration values of the phosphotungstic fraction converted into g. nitrogen give a more accurate value for the amino-nitrogen of that fraction.

In the filtrate fraction the titration value conveys nothing as to the exact state of the nitrogen in the monoamino-acids, since the nitrogen of a mono-amino-acid titrates quantitatively whether it is amino- or imino-nitrogen.

OTHER SOURCES OF ERROR IN THE VAN SLYKE METHOD.

It is well known that during the Kossel and Kutscher method of determining the diamino-acids when lysine is precipitated from the arginine- and histidine-free solution, there is more nitrogen present in the phosphotungstic precipitate than can be precipitated as lysine picrate even when the picrate is crystallised out in three or four successive isolations. There is no doubt that phosphotungstic acid precipitates small but appreciable amounts of monoamino-acids under such conditions. There is the same possibility in the Van Slyke method that some of the nitrogen precipitated is monoamino-acid nitrogen. Such nitrogen titrates quantitatively in alcoholic solution and is accounted for under lysine nitrogen in the distribution table. The amount of imino-monoamino-nitrogen is comparatively small in most proteins, and its possible precipitation would account for only a small fraction of the discrepancy between the results for observed amino-nitrogen and the titration equivalent of the amino-nitrogen of the bases mentioned above. Titration values throw no light in this direction.

SUGGESTED MODIFICATIONS OF THE VAN SLYKE METHOD FOR DETERMINING
THE DISTRIBUTION OF NITROGEN IN SEVEN GROUPS.

In view of the above work the following modifications of the Van Slyke method are tentatively suggested.

The complete hydrolysate. The arginine nitrogen is determined by the Plimmer method [1916] in an aliquot portion of the *original* hydrolysate, allowing for amide nitrogen driven off in the same process. The difference between the total nitrogen of the complete hydrolysate and the nitrogen equivalent to the alcohol-titration value of the same liquid is the non-titratable nitrogen and is equal to three-fourths of the arginine nitrogen plus two-thirds of the histidine nitrogen. From these two values the true arginine and histidine nitrogen values are obtained.

The phosphotungstic precipitate. The separation of the bases is carried out in the usual manner, the precipitate decomposed by the ether-amyl alcohol method and the alcohol titration value determined. The precipitated arginine and cystine are determined in the usual way, as well as the total diamino-nitrogen, so as to calculate the lysine nitrogen. The titration value converted to its equivalent weight of nitrogen is taken as the amino-nitrogen of the phosphotungstic precipitate. The precipitated histidine is calculated from the above data and the lysine determined by difference. The amounts of the precipitated arginine and histidine nitrogens are subtracted from the true values obtained from the complete hydrolysate and the differences, properly distributed into amino- and non-amino-nitrogen, allowed for in the two classes of nitrogen in the filtrate. The true arginine and histidine values are, of course, not corrected for solubility, and neither is the lysine nitrogen. Cystine nitrogen should be corrected for solubility according to the amounts laid down by Van Slyke [1912], but the amount of cystine present in ordinary proteins is too small for accurate determination by the distribution method, and no great stress can ever be put on the accuracy of this group.

The filtrate. This is best prepared for amino-nitrogen determination in the same way as the phosphotungstic precipitate, that is, the filtrate and washings from the precipitate are shaken up three times with ether-amyl alcohol mixture, the mixture being finally washed twice with dilute acid. The extracted solution is then evaporated to dryness *in vacuo* at 40°, dissolved in water and made up to 250 cc.

(Owing to nitrogenous impurities in some samples of amyl alcohol it will be found convenient to wash the half-and-half mixture of ether and amyl alcohol twice in a separating funnel with 5 % hydrochloric acid, or the mixture can be stored over 5 % acid and siphoned off as required. The nitrogenous bases, even in commercial amyl alcohol, can be completely removed by this method.)

The filtrate by this treatment can be titrated so as to check the titration value of the amino-acids precipitated by phosphotungstic acid. The quota of untitratable nitrogen in the filtrate can also be calculated and allowed for.

Practical results from the method.

Table IV sets out the results for gelatin as determined by the titration method compared with those obtained by the original Van Slyke method.

Table IV. *Results for gelatin by the titration method compared with those by the original Van Slyke method.*

	Percentage of total		
	Arginine	Histidine	Lysine
Nitrogen			
Titration method	17.23	2.58	5.71
Van Slyke method	14.70	4.48	6.32
	Percentage of protein		
	Arginine	Histidine	Lysine
Amino-acid			
Titration method	9.63	1.72	2.61
Van Slyke method	8.22	2.99	2.89
Kossel and Kutscher	9.3	0.4	2.8

For gelatin, closer values to the Kossel and Kutscher results are obtained by the modified method than by the original Van Slyke method.

SUMMARY.

Protein hydrolysates can be titrated to phenolphthalein in neutralised formalin-alcohol solution. The sum of the titration values for the amino-acid fractions separated by the Van Slyke method is equal to the titration value of the original hydrolysate.

By working in terms of nitrogen equivalent to the titratable value of solutions of amino-acid mixtures, much information can be obtained concerning the nitrogen distribution in such solutions.

The equivalent of the nitrogen of the monoamino-acid fraction should be quantitatively the same as the alcohol-titration value, but it has been found not to be completely represented by the titration value, this untitratable nitrogen representing three-quarters of the arginine and two-thirds of the histidine nitrogen not precipitated with phosphotungstic acid during the separation.

The amino-nitrogen of the diamino-acid fraction should be the equivalent of the titration value, but the titration equivalent has been found to be uniformly 22 % higher than the amino-nitrogen values determined by the nitrous acid method.

A modification of the Van Slyke method for determining the distribution of nitrogen in seven groups is suggested. This entails calculating the true values of the arginine and histidine nitrogens in the original hydrolysate, and the use of the titration value of the phosphotungstic fraction for calculating the amino-nitrogen content of that fraction.

In the practical application of the method, results more closely agreeing with the values found by the Kossel and Kutscher method are given for the diamino-acids.

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