The samples tested (all lately manufactured).

a. Polyzime "D" (dried Taka-Koji flour, Takamine Laboratory manufactured).
b. Polyzime (Taka-Koji extract, Takamine Laboratory manufactured).
c. Malt flour I.
d. Malt flour II.
e. Malt flour III.
f. Malt extract I.
g. Malt extract II.

	$D^{40°}_{30\ min.}$	= $D^{40°}_{2\ h.}$	= $D^{40°}_{24\ h.}$	= Lintner's value. 20°.	Lintner's value. 50°.
a	4700	24000	170000	96	250
b	3000	16000	115000	43	150
c	1053	4000	10000	156	550
d	1000	...	14000	139	410
e	850	...	15000	128	400
f	700	2700	6600	100	380
g	400	1000	8632	98	340

Summary.

1. The diastatic power of Polyzime does not decrease at temperatures lower than 40°. Below that temperature it preserves its enzymic activities for more than half a year with practically no change.

2. The optimum reaction of starch solution for liquefaction by Polyzime is neutral or very faintly acid.

3. Polyzime is 3 to 5 times stronger than ordinary malt extract in its amyloclastic power as indicated by testing according to Wohlgemuth's method.

4. The optimum temperature of starch liquefaction by Polyzime is 50° for a 30 minute to 2 hours digestion and 40° for 24 hours digestion, although it shows weaker saccharifying power than malt extract tested by Lintner's method.

CLIFTON, N. J.

[CONTRIBUTION FROM THE DEPARTMENT OF ANIMAL HUSBANDRY, UNIVERSITY OF ILLINOIS.]

THE ESTERIFICATION OF ALPHA AMINO ACIDS.

BY H. A. SHONLE AND H. H. MITCHELL.

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In the preparation of amino acids, or in the analysis of proteins by Fischer's ester method, some means of measuring the extent and rate of esterification would be of value. The rate of hydrolysis of a protein can be accurately followed by the Van Slyke amino nitrogen determination (as well as by other excellent methods), but in so far as we are aware, no accurate method has been devised for following quantitatively the subsequent esterification. Such a method would permit a direct determination of the relative merits of the different methods of esterification and

of the best conditions as regards temperature, time, concentration, and other factors under which such methods should be run. In the esterification of amino acid mixtures from proteins and of pure amino acids, much valuable information could be obtained as to the rate and extent of esterification. For example, are the di-amino acids as readily esterified as the monamino acids? Or, does the possibility exist that the diamino acids cannot be esterified by the ordinary procedure? Individual tests on amino acids and comparative tests on proteins containing different amino acids would be of value in clearing up this point.

Three methods have so far been used in following the rate of esterification, or in determining the amount of ester formed from organic acids, namely, (1) the changes in conductivity of the esterifying solution; (2) the actual isolation of the esters formed; and (3) the titration of the unesterified acidity.

Goldschmidt[1] has shown that, when such a catalyzer as picric acid or trichloroacetic acid is used in an absolute alcoholic solution of the organic acid, the change in conductivity due to the change in the concentration of the water is a satisfactory measure of the velocity of the esterification. While this method would give the velocity of the esterification, the actual amount of the ester formed could be determined only indirectly and with difficulty.

The isolation of the esters formed after a definite period of time, will, in the case of very stable esters, give accurate results. Phelps and Hubbard[2] used this method very successfully with succinic acid. The succinic ester, after being first freed from traces of acid by treatment with a solution of sodium carbonate, was extracted with ether and distilled under diminished pressure. The accuracy of this method depends upon the stability of the ester and its solubility in organic solvents.

All of the investigations on the esterification of α-amino acids have heretofore depended upon the actual isolation of the α-amino acid ester, yet these esters are quite readily saponified. Investigations, carried out by Abderhalden and Weil,[3] show that there is no difference in the yield of glutamic and aspartic acids when their ester hydrochlorides are freed by sodium hydroxide, sodium ethylate, or ammonia. Using Fischer's method of esterification, they isolated and distilled 64.7 to 81.3% of the esters of aspartic acid and 65 to 77.5% of the esters of glutamic acid. The crude esters cannot be weighed as such, since they always contain impurities and, when distillation is the means of purification, there is always an undistillable residue left representing decomposition products of the esters.

Osborne and Jones[4] modified the regular Fischer method by using the

[1] Goldschmidt, *Elektrochem. Z.*, 15, 4 (1909).

[2] Phelps and Hubbard, *Am. J. Sci.*, 23, 368 (1907).

[3] Abderhalden and Weil, *Z. physiol. Chem.*, 74, 445 (1911).

[4] Osborne and Jones, *Am. J. Physiol.*, 26, 212 (1910).

Phelps and Hubbard[1] method of esterifying organic acids. They recovered 88.8% of leucine, 85% of glutamic acid and 69.5% of proline from one esterification of weighed amounts of these amino acids. These yields are somewhat higher than those usually secured and this increase is attributed to the superiority of the Phelps and Hubbard method. Levene and Van Slyke[2] similarly were able to recover 92% of valine in the form of its constant boiling ester, using the Fischer method and liberating the ester hydrochloride with barium hydroxide. Osborne and Jones[1] found that 75% of an amino acid mixture is converted into esters at each esterification, calculating on the basis of the esters actually isolated. Many other instances can be cited, all of which show a varying and incomplete esterification.

In all the work of Abderhalden, and Osborne and Jones, on α-amino acids, it must be remembered that the yields of esters obtained are influenced by the incomplete recovery of the esters, due to the unavoidable loss of the esters saponified during the liberation of their hydrochlorides, and due to the inability of the solvent to extract the esters completely from the pasty mass.

In most of the work done in securing the different constants in the esterification of organic acids, the rate of esterification has been followed by titrimetric methods. Goldschmidt,[3] Goldschmidt and Sunde,[4] Kailan,[5] Thomas and Sudborough,[6] and many others, have followed this method with satisfactory results. The method itself is quite simple. Definite amounts of alcohol, organic acid and catalyzer are placed in a sealed vessel in a thermostat and aliquot portions are taken out for analysis from time to time. Either 0.2 or 0.1 N barium or sodium hydroxide solution is used for the titration with phenolphthalein as an indicator. In cases where the esters are readily saponified, the titration is carried out with ammonium hydroxide and litmus. When hydrochloric acid is used as a catalyzer, the mineral acid acidity can readily be determined from the amount of chlorine present as determined by a Volhard titration.

That this method is best adapted for quantitative work is without question, since it allows the determination, at any period in the process of esterification, of the per cent. of unesterified acidity. In order to apply this method successfully to the esterification of α-amino acids, some means must be taken to overcome the basic influence of the α-amino group, since α-amino acids themselves cannot be determined by titration.

[1] *Loc. cit.*

[2] Levene and Van Slyke, *J. Biol. Chem.*, 6, 479 (1909).

[3] Goldschmidt, *Ber.*, **28**, 3218 (1895).

[4] Goldschmidt and Sunde, *ibid.*, **39**, 711 (1906).

[5] Kailan, *Monatsh.*, **27**, 542 (1906).

[6] Thomas and Sudborough, *Proc. Chem. Soc.*, **27**, 314 (1912).

The purpose of the investigation reported in this paper was to modify the titrimetric method in order to adapt it to the determination of the esterification of α-amino acids. The modification used involves the use of the Sörensen formaldehyde titration of amino acids, by which the amino acid acidity is titrated after the destruction of the amino group by condensation with formaldehyde. The technique of the method is described in great detail by Sörensen,[1] both for colorless or colored solutions of amino acids and amino acid mixtures.

Obviously the presence of amino acid esters would not influence the Sörensen titration of the free amino acids, provided that this titration could be conducted in such a way as to avoid any considerable saponification. In obtaining the per cent. of unesterified amino acid acidity at any stage of the process of esterification, samples were withdrawn from the esterifying mixture from time to time and made up to volume. The unesterified amino acids were determined by running a Sörensen titration on an aliquot portion, and deducting the mineral acidity as determined in a second aliquot portion by methods which will be discussed below. The total amino acid acidity was then determined in another aliquot portion. Thus, the per cent. of unesterified amino acid acidity could be calculated from these data by employing the proper correction for volume.

The experimental work of this investigation was conducted mainly on mixtures of amino acids as obtained by hydrolyzing protein, generally casein, with 20% hydrochloric acid, until the Van Slyke amino nitrogen determinations indicated complete hydrolysis. The hydrolyzate was then prepared for esterification in the usual way, after the partial removal of the glutamic acid (usually) and the subsequent removal of the excess hydrochloric acid and water by repeated evaporation with absolute alcohol.

The resulting deeply colored, thick syrup was esterified by the method of Phelps and Hubbard[2] as applied to amino acid mixtures by Osborne and Jones.[2] This method of esterification consists in heating the absolute alcoholic solution of the amino acid hydrochlorides and the residual free hydrochloric acid to 100 to 105° in an oil bath, and passing vapors of a boiling 2% solution of alcoholic hydrochloric acid in absolute alcohol through the mixture. The alcoholic hydrochloric acid was made by saturating absolute alcohol with dry hydrogen chloride. Hydrochloric acid was the only catalyzer used. Under these conditions of esterification the water formed during the reaction is presumably removed as fast as formed. At definite intervals during the esterification, 3 to 5 cc. samples of the solution were removed for analysis by means of a pipet.

[1] Sörensen, *Biochem. Z.*, **7**, 47, 407 (1907–08).

[2] *Loc. cit.*

During the course of the investigation many procedures were tested for determining the mineral acidity, for decolorizing the samples, etc., which were afterwards discarded for better ones. A brief description of the experience obtained in the course of the work will illustrate the many difficulties that had to be overcome before a fairly successful method was finally elaborated.

At first the samples removed for analysis were diluted with water and decolorized by the addition of 20 to 30 cc. of aqueous silver nitrate solution (about 0.3 *N*) as recommended by Sörensen. Care was taken that the solution still contained chlorides after this treatment. The silver chloride formed carried down most of the soluble coloring matter. The sample was then made up to a definite volume, usually 100 cc. filtered through a dry filter and aliquot portions submitted to a series of titrations.[1]

The total acidity of the sample was determined by a Sörensen titration measuring, (1) the free amino acid acidity, (2) the hydrochloric acid acidity, both that portion originally uncombined and that portion liberated by the condensation of the formaldehyde with the amino groups and with any ammonium chloride present, and (3) the nitric acid acidity added with the silver nitrate.

The total mineral acid acidity present after treatment with formaldehyde was taken as the sum of (1) the total hydrochloric acid acidity as determined by a Volhard chlorine determination, and (2) the total nitric acid acidity as determined from the amount of silver nitrate added during the decolorization of the solution. This determination is based on the assumption that all the mineral acid, free and combined, will be in the uncombined state after treatment with formaldehyde, an assumption which Sörensen found to be correct.

The difference between the value of the Sörensen titration and the determination of the mineral acids by the latter method is a measure of the free amino acid acidity.

The total amino acid acidity was determined by running a Sörensen titration on an aliquot portion of the filtrate which had been completely saponified by boiling with water and dil. hydrochloric acid for 18 hours under a reflux condenser.[2]

[1] The end-point of the Sörensen titration corresponds to an excess of alkali. Obviously any free alkali is to be avoided when esters are present. Consequently in all the titrations carried on in the presence of esters, the first distinct pink color which corresponds to Sörensen's "second stage" had to be used as the end-point instead of the deep red "third stage." For this same reason it was impossible to add an excess of alkali and titrate back with acid as in the original procedure. The use of this "second stage" gives a value averaging 6 to 8 % below the actual amino acid content of protein hydrolyzates, as can be calculated by applying Sörensen's tables to the analysis of proteins.

[2] Saponification by means of barium or sodium hydroxide undoubtedly would be

The mineral acid acidity after saponification could not be determined as before saponification, due to the loss of nitric acid during refluxing. It was therefore determined by a titration to litmus as in the ordinary Sörensen procedure. The 2 methods are comparable except for the presence of the dibasic amino acids and ammonium chloride. The titration to litmus would include half the acidity of the former, and none of the hydrochloric acid contained in the latter. The mono-basic amino acids would not be titrated at all, though the hydrochloric acid combined with them would be titrated.

From the total amino acid acidity, as determined by the difference between these 2 titrations on the saponified solution, and the free amino acid acidity as determined on the original sample, the extent of esterification was calculated.

The results obtained from the above methods were not particularly encouraging. The percentage of the unesterified amino acid acidity during the course of 6 to 8 hours esterification exhibited unaccountable fluctuations. It was thought that saponification probably took place during the titration of the aqueous solution of the esters with barium hydroxide. Consequently a series of tests was carried out with amino acid esters obtained from casein. After standing for 18 hours in alcohol of various strengths, the ester solutions were titrated. No saponification could be detected in the 95 and 80% alcoholic solutions. In the 60% alcohol, 6.4% of the ester was saponified, while in the aqueous solution a 25% saponification resulted. When a Sörensen titration was run on identical amounts of amino acid esters diluted with water, 55, 75 and 95% alcohol, the amount of saponification increased with the increasing per cent. of water. Redistilled alcohol was therefore substituted for water as a diluent. The titrations were carried out with alcoholic silver nitrate and sodium hydroxide.

Several esterifications of the mixtures of amino acids resulting from the hydrolysis of 175 to 200 g. of casein were followed by the method

quicker, but there is a danger of the decomposition of the di-amino acids, if present. Also, when tests were run with barium hydroxide as a saponifying agent, a precipitate was always secured which was soluble in hydrochloric acid, and was presumably barium carbonate. Furthermore, glutamic acid is readily converted into pyrrolidone carboxylic acid by boiling in the presence of alkali or by long continued boiling in water. (Foreman, *Biochem. J.*, **8**, 481 (1914).) The esters of glutamic acid (and aspartic acid by analogy) can be saponified by boiling with hydrochloric acid without the intermediate formation of pyrrolidone carboxylic acid. (Abderhalden and Weil, *Z. physiol. Chem.*, **74**, 445 (1911).) The other amino acids found in proteins are comparatively readily saponified by boiling with water alone as has been shown by Fischer.

After the acid saponification, a finely divided black precipitate was formed. This was removed by filtering the sample, after it had been made up to a definite volume, through a dry filter. No attempt was made to discover the nature of this precipitate which was negligible in amount.

modified as indicated above. The results are included in Table I. During the time of esterification, 1600 to 1800 cc. of absolute alcoholic hydrochloric acid were passed through the mixture of amino acid hydrochlorides, alcohol and free hydrochloric acid. The bath was kept at a temperature of 110°.

TABLE I.

Expt. 1. Unesterified amino acid acidity. %	Time in hours.	Expt. 2. Unesterified amino acid acidity. %
63	0.00	64
37	0.50	41
33	1.00	..
..	1.25	36
37	2.00	..
..	2.25	35
33	3.00	..
31	4.00	32
27	5.00	..

The above experiments show that 36% of the amino acid acidity was esterified during the repeated evaporation with absolute alcohol in removing the water. Most of the subsequent esterification occurred during the first hour, and from then on there was a slow and somewhat regular decrease of the unesterified acidity. The slowing up of the esterification was probably due to the difficulty in removing the small amounts of water formed during the esterification by means of alcohol. Considerable trouble was experienced in efficiently decolorizing the samples by means of the silver nitrate method with the result that color formation at the end point of the Sörensen titrations was somewhat masked.

An experiment was carried out on a mixture of amino acids resulting from the hydrolysis of 200 g. of casein, from which the glutamic and aspartic acid had been removed by the method of Foreman.[1] During this procedure an excess of calcium hydroxide was added to the hydrolyzate and the mixture filtered. The filtrate containing the remaining amino acids was light yellow in color, the melanin having been removed by the lime. The calcium was removed and the filtrate was prepared for esterification as usual. About 1500 cc. of alcoholic hydrochloric acid was passed through the mixture of amino acid hydrochlorides. The results are given in Table II.

As it was not necessary to add silver nitrate for decolorization (the mixture remaining clear throughout the run), the mineral acid acidity was entirely due to hydrochloric acid and was determined both before and after saponification by a Volhard chlorine titration. This simplified the procedure and made the determination before and after saponification comparable.

[1] Foreman, *Biochem. J.*, 8, 463 (1914).

TABLE II.

Unesterified amino acid acidity. %	Time in hours.
75	0.00
56	0.50
40	1.00
47	2.00
43	4.00
33	6.00

Since the method of decolorization involving the use of silver nitrate was not found to be efficient, and since it complicated the determination of the mineral acid acidity after saponification, a method which would decolorize the entire solution before esterification without the addition of any inorganic salts or mineral acids other than hydrochloric would be very advantageous. The calcium hydroxide, used in the last experiment, while efficient in decolorizing, was difficult to remove quantitatively.

Denis[1] found that the addition of potassium alum to a colored hydrolyzate made alkaline with sodium carbonate, almost completely decolorized the solution. The soluble coloring matter was carried down with the precipitate of aluminum hydroxide formed. Instead of potassium alum and sodium carbonate, aluminum sulfate and barium hydroxide were used, because they can be quantitatively removed from the solution. The hydrolyzate, after almost all of the hydrochloric acid had been removed by evaporation *in vacuo*, was made slightly alkaline with barium hydroxide. Ten g. of aluminum sulfate was then added for each 100 g. of protein used. The precipitate of aluminum hydroxide, barium sulfate and melanin was filtered off, leaving a clear yellow filtrate which was free from aluminum. To remove the barium quantitatively as the sulfate, it was found necessary to add a slight excess of sulfuric acid and heat the solution to boiling. All the barium was then precipitated as the sulfate, and the excess of acid could be exactly removed by adding barium hydroxide in the hot. The filtrate was then prepared for esterification as usual.

Three esterifications of mixtures of amino acids from, (1) 50 g. of casein, (2) 25 g. of vignin, and (3) 50 g. of gelatin, were followed by the method modified as indicated above. The procedure in each case was practically the same in regard to time, temperature, etc. The temperature of the oil bath was kept sufficiently high (about 120°) so that the temperature of the esterifying mixture was between 100 and 105°. In each case one liter of 2% alcoholic hydrochloric acid was passed through at a constant rate for 8 hours.

[1] Denis, *J. Biol. Chem.*, **8**, 431 (1910).

The usual samples were taken off at definite intervals and made up to 50 cc. with redistilled 95% alcohol. A 10 cc. portion was used for a Sörensen titration and a 5 cc. portion for a Volhard chlorine determination. Twenty-five cc. was saponified by boiling with dil. hydrochloric acid for a period of 18 hours under a reflux condenser. The saponified sample was then evaporated on the steam bath to a small volume and made up to the original volume of 25 cc. A 10 cc. portion of this was used for a Sörensen titration, and a 5 cc. portion for a Volhard chlorine determination. By applying the proper volume corrections, the differences between the titrations before saponification will give the free (unesterified) amino acid acidity, while those after saponification will give the total amino acid acidity in terms directly comparable to the first titration. From these 2 values, expressed in cc. of N acid, the per cent. of unesterified amino acid acidity can be determined directly. The standard solutions used for the Sörensen titration, however, were 0.2 N and those for the Volhard titration were 0.1 N. The results of this series are given in Table III in terms of N acid.

TABLE III.

Expt. 1, Casein.

Before Saponification.

Time in hours	0.00[a]	0.50	1.50	3.50	5.50	8.00
Total acid	....	2.965	3.336	2.586	4.141	3.434
Mineral acid	....	2.213	2.562	1.995	3.520	2.850
Free amino acid	....	0.752	0.594	0.591	0.621	0.584

After Saponification.

Time in hours	0.00[a]	0.50	1.50	3.50	5.50	8.00
Total acid	....	4.861	5.958	5.073	5.604	5.954
Mineral acid	....	3.385	4.147	3.494	3.832	3.543
Total amino acid	....	1.476	1.811	1.579	1.772	2.411
Unesterified amino acid acidity, %		51	43	38	36	24

Expt. 2, Vignin.

Before Saponification.

Time in hours	0.00	0.50	1.50	3.50	5.50	8.00
Total acid	2.809	2.311	3.369	2.277	3.792	2.155
Mineral acid	1.704	1.894	2.794	2.050	3.420	1.902
Free amino acid	1.105	0.417	0.575	0.227	0.372	0.253

After Saponification.

Time in hours	0.00	0.50	1.50	3.50	5.50	8.00
Total acid	6.877	6.076	5.221	3.046	6.346	3.213
Mineral acid	5.690	4.702	3.302	1.866	4.002	1.934
Total amino acid	1.187	1.374	1.919	1.180	2.344	1.279
Unesterified amino acid acidity, %	93	30	30	19	16	20

[a] The sample taken off at the start gave an impossible result.

Expt. 3, Gelatin.

Before Saponification.

Time in hours	0.00	0.50	1.50	3.50	5.50	8.00
Total acid	4.780	5.412	4.402	3.036	4.166	3.491
Mineral acid	2.860	4.018	3.431	2.474	3.508	3.018
Free amino acid	1.920	1.394	0.971	0.562	0.658	0.473

After Saponification.

Time in hours	0.00	0.50	1.50	3.50	5.50	8.00
Total acid	8.144	9.125	7.962	4.227	6.350	5.410
Mineral acid	5.485	6.182	4.695	2.464	3.798	3.199
Total amino acid	2.659	2.943	3.267	1.763	2.552	2.211
Unesterified amino acid acidity, %	72	46	30	32	26	21

No perceptible deepening of the color of the mixture was notice during the course of esterification. The slightly higher results obtained for the extent of the esterification, as compared with the values obtained in the previous experiments, may be attributed to the slightly higher temperature of the esterifying mixture and the larger proportional amount of alcohol passed through it. From the results shown in Table III, it is evident that the percentage of unesterified amino acid acidity was slowly decreasing at the end of 8 hours. It is probable that the period of esterification used in the work of Osborne and associated (6 to 8 hours) is not sufficiently long to secure maximum results.

The rate of esterification of the casein was somewhat slower than that of the gelatin or vignin, but in the case of the vignin it must be remembered that twice as much alcohol was passed through the esterifying mixture in proportion to the amount of protein used, as in the other cases. At present we are unable to say, because of too little evidence, whether or not qualitative differences in amino acid mixtures will cause definite differences in the rate of their esterification. However, the absence of striking differences in the figures obtained from such different proteins as casein, vignin, and gelatin is significant. The fact that for each protein the extent of esterification at the end of 8 hours is approximately the same is noteworthy, and would indicate that the unesterified amino acid acidity represents an approach to an equilibrium rather than to a definite unesterifiable fraction.

It was found possible to esterify lysine to the extent of 82% in several hours time. Alanine could be esterified to the extent of 96% and glutamic acid 85%. These values are not to be regarded as being maximal in the case of any of the above amino acids. From this we can see that the 3 groups of amino acids present in proteins are esterifiable.

In carrying out the analysis of casein by the Fischer method, several fractions of the ether insoluble residues were secured. These residues very largely consist of the di-amino acids. Upon subjecting these amino acids to the regular procedure for esterification for 20 hours, the following results were secured:

TABLE IV.
Di-amino Acid Fraction.

Time in hours.	Unesterified amino acid acidity. %
0.00	50
1.00	45
2.00	43
7.00	38
11.50	27
15.50	26
20.50	11

A comparison with Tables I, II and III shows that the rate of esterification was slower when the di-amino acids were present in a large concentration.

Several samples of amino acid mixtures from casein were esterified for 20 to 24 hours. In one case it was found possible to reduce the unesterified residue of amino acids to 12%. Unexplainable fluctuations occurred in the percentage of unesterified amino acid acidity during the last 12 hours of esterification. We are planning a series of experiments to determine whether there is a real fluctuation in the actual amount of ester present or whether, due to a possible heterogeneous condition of the syrupy esterification mixture, our method of sampling was at fault. Thus when an amino acid mixture from vegetable albumin was subjected to an esterification for 18 hours, the following results were secured:

TABLE V.

Time in hours.	Unesterified amino acid acidity. %
12.0	13
15.0	9
18.0	21

Possibly during the later stages of the esterification as the mixtures become more and more dehydrated, the unesterified amino acids crystallize out of solution and interfere seriously with the sampling.

The accuracy of the above method of determining the extent and the rate of esterification of α-amino acids depends, (1) on the accuracy of the quantitative methods used; (2) on the stability of the amino acid esters during the Sörensen titration; and (3) on the homogeneity of the mixture from which the samples are taken.

The methods used are in themselves accurate for the determination of the ratio of unesterified amino acid to total amino acid content. As shown above, the amino acid esters are stable in 95% alcohol, but they are readily saponified at the concentration of hydroxyl ion required in the Sörensen titration.

The per cent. of amino acid esters saponified during a Sörensen titra-

tion decreases with the increasing concentration of the ester solution. The extent of this is shown in Table VI.

TABLE VI.

Normality of amino acid ester in sample titrated.	Ester saponified during titration. %
0.71	1.4
0.25	2.4
0.06	7.7
0.04	8.4
0.02	14.6

The amino acid ester concentration in the samples subjected to the Sörensen titration in our procedure varied from 0.1 to 0.2 N (see Table III). Accordingly, therefore, a saponification of 5 to 3%, occurred during the titration. Consequently the amount of amino acid acidity titrated is the sum of the unesterified amino acids present in the sample and of that fraction formed by the saponification of the esters during the operation.

However, since the Sörensen titration is discontinued at the "second color stage," the titrations secured are, as shown above, 6 to 8% low. Thus the 3 to 5% of saponification of the esters (in which state 80 to 90% of the total amino acid content of the sample is represented) will be only partially offset by the 6 to 8% undertitration of the amino acids. From these data, the titration of a sample containing 90% of the amino acids as esters will, by the methods used, show an ester content 2 to 3% below the amount actually present. Consequently the reported values of the free amino acid acidity of samples removed near the completion of the esterification are several per cent. high.

Summary and Conclusion.

The rate and extent of esterification of organic acids can be conveniently followed by titrametric methods. In adapting such a method to the esterification of α-amino acids, some means must be used to overcome the basicity of the amino group before the free amino acid acidity can be titrated. The modification employed involves the use of the Sörensen titration of amino acids. The free amino acid acidity in samples withdrawn from the esterification flask, is titrated with the free mineral acid acidity after the destruction of the amino groups and ammonium salts with formaldehyde. The determination of the free and combined hydrochloric acid (the only mineral acid present) is effected by a Volhard chlorine titration. The difference between these titrations represents the free amino acid acidity. The same procedure applied to an aliquot portion of the same sample after saponification with dil. hydrochloric acid, will give the total amino acid acidity. From these 2 results, the per cent. of unesterified amino acid can be readily calculated.

In the case of highly and colored mixtures of amino acids from protein hydrolysis, decolorization was effected by the addition of aluminum sulfate to the hydrolyzate made alkaline with barium hydroxide and subsequent filtration. The barium was removed as the sulfate.

The method has been successfully applied to mixtures of amino acids from casein, vignin, gelatin and vegetable albumin and to mixtures rich in the di-amino acids, as well as to individual amino acids, and shows that in a single esterification as high as 90% of the amino acid acidity may be esterified in the case of amino acid mixtures. Alanine was 96% esterified, lysine 82% and glutamic acid 85%. From the data given a tentative correction of 2 or 3% can be added to the per cent. of amino acid esterified.

From the results obtained in this investigation, it appears that there is no unesterifiable residue of amino acids. The reaction as carried out by the above method is apparently one in which an equilibrium is reached when an appreciable amount of amino acid is still unesterified.

This method will be of value in determining the best conditions under which the esterification of α-amino acids should be run, and in comparing the efficiency of the different methods of esterification.[1]

URBANA, ILL.

THE COMBINATION OF FRACTIONATION WITH SPECTROPHOTOMETRY IN PROXIMATE ORGANIC ANALYSIS.[2]

BY W. E. MATHEWSON.

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In making the chemical examination of various commercial products it is often desirable to estimate with some precision certain colorless substances which are found in quantities of less than a few mg. in the sample available. The spectrophotometric method[3] for estimating dyes has the special advantages of being applicable with very small amounts of the coloring matters and requiring no quantitative preliminary purification of them. It has not been largely used for the determination of colorless organic compounds because but few of these substances can be converted easily and completely into colored derivatives by means of reagents that are themselves colorless or widely different in shade. A procedure of somewhat general applicability, however, consists in combining or condensing the substance to be estimated with a suitable compound

[1] An investigation of the cause of the variation of the amount of esters formed during long continued esterification is being planned. The method will also be so modified as to allow the addition (or subtraction) of a correction factor which will include the under-titration as well as the saponification of the amino acid esters.

[2] Published by permission of the Secretary of Agriculture.

[3] For the development of quantitative spectrophotometry, see G. and H. Kruess, "Kolorimetrie und quantitativ Spektralanalyse," Hamburg, 1909.