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On The Esterification Of Amino Acids.

ON THE ESTERIFICATION OF AMINO ACIDS

BY

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THESIS

FOR THE

DEGREE OF BACHELOR OF SCIENCE

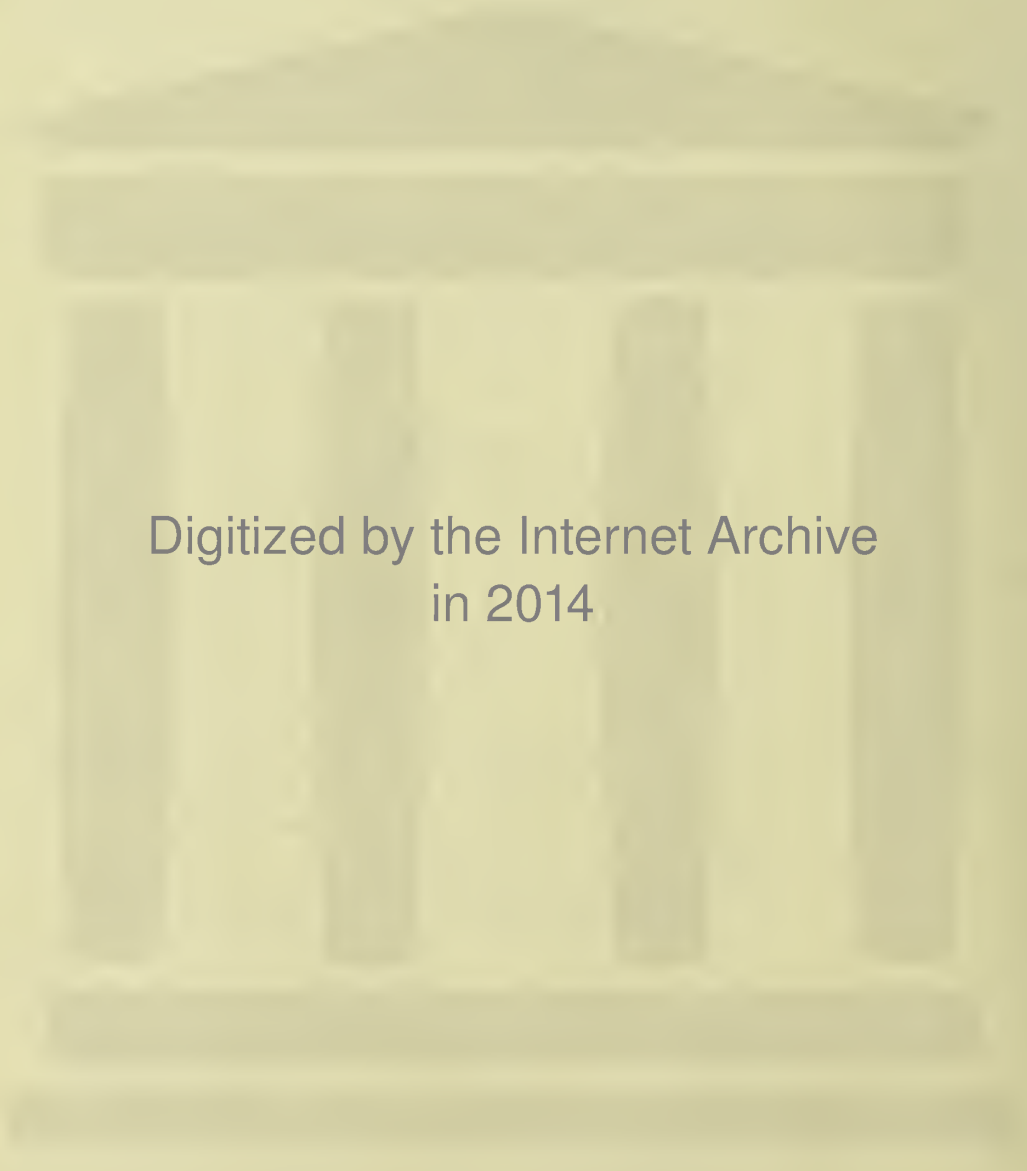
IN

CHEMISTRY

COLLEGE OF LIBERAL ARTS AND SCIENCES

UNIVERSITY OF ILLINOIS

1916



UNIVERSITY OF ILLINOIS

May 31, 1916

THIS IS TO CERTIFY THAT THE THESIS PREPARED UNDER MY SUPERVISION BY

Horace Abbott Shonle

ENTITLED On the Esterification of Amino Acids

IS APPROVED BY ME AS FULFILLING THIS PART OF THE REQUIREMENTS FOR THE

DEGREE OF Bachelor of Science in Chemistry.

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ON THE ESTERIFICATION OF AMINO ACIDS.

In the preparation of amino acids or in the analysis of proteins by the Fischer ester method, a 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 method (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 test to determine the relative merits of the different methods of esterification and to determine the best conditions as regards temperature, length of time, concentration and other factors under which such methods should be run. In the esterification of amino acid mixtures from proteins and pure amino acids, much valuable information could be obtained as to the rate and extent of esterification. For example, we have been unable to find in the literature any positive information as to whether or not the diamino acids are esterifiable. Therefore, it cannot be at present said that all the amino acids are esterifiable and the possibility exists that a certain fraction of the amino 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 been used in following the rate of esterification or in determining the amount of ester formed from organic acids, namely,- the changes in conductivity of the esterifying solution, the actual isolation of the esters formed, and

the titration of the unesterified acidity.

Goldschmidt[I] has shown that, when such a catalyzer as picric or trichloroacetic acids 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 was found to be an efficient measure of the velocity of the esterification. While this would give the velocity of the esterification easily, the actual amount of ester formed could only be determined 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 sucessfully 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 on the stability of the ester and its solubility in ether. Sudborough and Lloyd[3], V.Meyer[4] and Fischer and Spier[5] were among those who used this method.

All of the investigations on the esterification of amino acids have been carried out by this method, yet amino acid esters are quite readily saponified. Investigations, carried out by Abderhalden and Weil[6], show there is no difference in the yield of glutamic and aspartic acids when freed by sodium hydroxide, sodium ethylate or ammonia. Using the Fischer method of esterification, they isolated and distilled 64.7 to 81.3 percent of the esters of aspartic acid and 65 to 77.5 percent 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[7] modified the regular Fischer method by using the Phelps and Phelps[8] method of esterifying organic acids. They recovered 88.8 percent of leucine, 85 percent of glutamic acid and 69.5 percent of proline after esterifying 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 Phelps method. Levene and Van Slyke[9] were able to recover 92 percent of valine in the form of its constant boiling ester, using the Fischer method and liberating the ester hydrochlorides with barium hydroxide. Osborne and Jones[7] found that 75 percent 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 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 incomplete extraction of the esters by the solvent used.

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 titrametric methods. Goldschmidt[10], Goldschmidt and Sunde[11], Kailan[12], Thomas and Sudborough[13] 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 fifth or tenth normal barium or sodium hydroxides are 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 calculated 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 of the process, of the esterification of the percent of unesterified acidity. In order to apply this method successfully to the esterification of alpha amino acids, some means must be taken to overcome the basic influence of the alpha amino group, since alpha amino acids themselves cannot be determined by titration.

The purpose of the investigation reported in this paper was to modify the titrametric method in order to adapt it to the determination of the esterification of alpha 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 the formaldehyde. The technique of the method is described in great detail by Sörenser[14], both for colorless or colored solutions of amino acids or 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 percent of unesterified amino acid acidity at any stage in 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 determined in another aliquot portion, at first by a Van Slyke amino nitrogen determination, but later by a second Sörensen titration after saponification by boiling for several hours with water and hydrochloric acid and by deducting the acidity due to the mineral acids. Thus, the percent of unesterified amino acid acidity could be calculated from these data by employing the proper volume corrections.

The experimental work of this investigation was conducted mainly on mixtures of amino acids as obtained by hydrolyzing proteins, generally casein, with twenty percent 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 as the hydrochloride (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[6] as applied to amino acid mix- by Osborne and Jones[7]. 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 degrees on an oil bath and passing the vapors of a boiling two percent solution of alcoholic hydrochloric acid in absolute alcohol through the mixture. The alcoholic hydrochloric acid was made by saturating absolute alcohol with dry hydrochloric acid gas, secured from the action of concentrated sulfuric acid on concentrated hydrochloric acid. 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. In the first work with this method, due to a misreading of the published directions, the esterification mixture was heated in an oil bath kept at 100 to 105 degrees instead of being itself heated to that temperature. At definite intervals of time during the esterification, 3 to 5 cc samples of the solution were removed for analysis by means of a pipette.

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 third normal) 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, and filtered through a dry filter. A 10 cc portion of the filtrate was then submitted to a Sörensen titration. In the Sörensen titration, the end point corresponds to an excess of alkali. Obviously any free alkali is to be avoided when esters are present, and hence in all the titrations carried out in the presence of esters, the first pink color was used as an end point instead of the deep red. The Sörensen titration determines the total acidity of the sample, i.e. the amino acid acidity, the hydrochloric acid acidity, both that portion originally uncombined and that portion liberated by the condensation of the formaldehyde with the amino groups and any ammonium chloride present, and the nitric acid acidity added with the silver nitrate. The mineral acid acidity was then determined in another aliquot portion by titration with barium hydroxide with phenolphthalein as an indicator, and the difference between these two titrations was taken as a measure of the free amino acid acidity. The total amino acid acidity was determined by running a Van Slyke amino nitrogen determination on a third aliquot portion of the filtrate and assuming that one atom of alpha amino nitrogen was equivalent to one carboxyl group.

The results obtained by the above method were not particularly encouraging. The percentages of unesterified amino acid acidity during the course of six to eight hours esterification exhibited unaccountable fluctuations. It was thought probable that

saponification took place on dilution of the samples with water and during the titration with the aqueous barium hydroxide solution. Redistilled 95 percent alcohol was therefore substituted for water as a diluent. Also alcoholic silver nitrate and sodium hydroxide solutions were used. Before making a Van Slyke determination on a portion of the filtrate, the alcohol was evaporated off and the residue taken up with water.

In comparing the modified procedure with the original, it was shown that the substitution of alcohol for water reduced the saponification and on testing the two methods with an alanine esterification, better results were obtained with the modified than with the original procedure. Later tests with mixtures of amino acid esters, showed that they were extremely stable in 95 percent alcohol solution in the absence of free alkali, even after eighteen hours standing, while in water solution an appreciable saponification resulted. However, the modified method was not successful when applied to protein hydrolyzates because the fluctuations while less were still noticable. The trouble was finally located in the determination of the mineral acid acidity and the total amino acid acidity.

Titration of a mixture of free hydrochloric acid, amino acid hydrochlorides, amino acid ester hydrochlorides and ammonium chloride, using phenolphthalein as an indicator was found to be inaccurate as a measure of the free and combined mineral acidity for the following reasons:- (1) a small part of the free amino acid acidity would be titrated, (2) due to the fact that the amino acid esters are very basic, a part of the hydrochloric acid com-

bined with them could not be titrated with phenolphthalein, and (3) none of the hydrochloric acid combined with ammonia could be titrated. For these reasons, the total mineral acid acidity which could be titrated after treatment with formaldehyde was taken as the sum of, (1) the total hydrochloric acid acidity as determined by a Volhard chlorine determination[i], and (2) the total nitric acid acidity as determined from the amount of silver nitrate added during the decolorization of the solution.

[i] In comparing the amount of hydrochloric acid present in a sample of alcoholic hydrochloric acid as determined by the Volhard method and by titration with Congo red as an indicator, close checks were secured. Evidently the presence of ethyl chloride which is formed in small amounts during the esterification does not invalidate this method of determining chlorine.

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.

As is well known, the Sörensen and Van Slyke determinations are not strictly comparable[i].

[i] The amino acid acidity of a mixture of amino acids from casein was 7 percent greater as determined by the Sörensen method than by the Van Slyke, assuming that one atom of alpha amino nitrogen was equivalent to one carboxyl group. White and Thomas (Journ. Biol. Chem., 13, III-16) found that the amino acid acidity in the case of tryptic hydrolysis of flesh was greater when determined by the Sörensen method than when determined by the Van Slyke method.

Therefore, it was decided to determine the total amino acid acidity by saponifying an aliquot portion of the filtrate by boiling with

water and hydrochloric acid several hours under a reflux condenser, after the alcohol had been evaporated off at a low temperature[1].

[1] Saponification by means of barium or sodium hydroxides undoubtedly would be quicker, but there is the danger of decomposition of the diamino acids if present. Also when tests were run with barium hydroxide, 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 with water. (Foreman, Biochem. Journ., 8, 481.) 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,Zeit. f. Physiol. Chem.,74, 445.) The other amino acids found in proteins are comparatively readily saponified by boiling with water alone as shown by Fischer.

The solution was then made up to volume and a Sörensen titration run on an aliquot portion to give the total acidity. The mineral acid acidity in this case could not be determined as before saponification (see preceding paragraph), 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 two methods are strictly comparable except for the presence of the dibasic acids. The titration to litmus would include half of the acidity of these acids. The monobasic 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 two titrations on the saponified solution and the free amino acid acidity as determined on the original sample, the extent of esterification was calculated.

Several esterifications of the mixtures of amino acids resulting from the hydrolysis of 175 to 200 grams of casein were fol-

lowed by the method modified as indicated above. The results are included in table I. During the time of the esterification, I600 to I800 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 II0 degrees.

TABLE I.

Experiment I.			Experiment II.	
Percent of unesterified amino acid acidity.		Time in hours.	Percent of unesterified amino acid acidity.	
63.4	----------	0.00	----------	64.I
36.5	----------	0.50	----------	4I.2
32.6	----------	I.00	----------	
		I.25	----------	36.3
36.7	----------	2.00	-	
		2.25	----------	35.0
32.8	----------	3.00		
3I.2	----------	4.00	----------	32.2
26.8	----------	5.00		

The above experiments show that 36 percent of the amino acid acidity was esterified during the repeated evaporation with absolute alcohol in order to remove the water. Most of the esterification occured during the first hour, from then on there is a slow and somewhat regular decrease of the unesterified acidity. The slowing up of the esterification is probably due to the difficulty in removing the small amounts of water formed by means of alcohol. Considerable trouble was experienced in sufficiently decolorizing the samples by means of the silver nitrate method so that a sharp end point could be secured in the Sörensen titration.[i]

[i] In running the Sörensen titration in alcoholic solutions, the correction factor secured from the control solution (which is subtracted from the total amount of alkali used) was found to vary and increased proportionally with

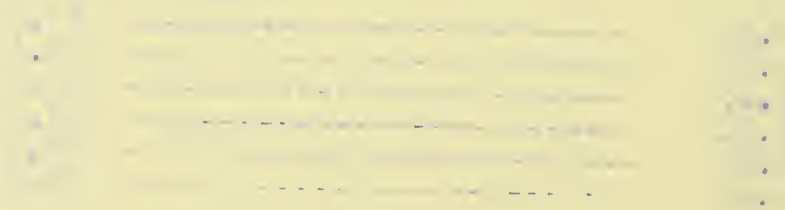
the amount of sodium hydroxide required.

A third experiment was carried out on a mixture of amino acids resulting from the hydrolysis of 200 grams of casein, from which the glutamic and aspartic acids had been removed by the method of Foreman[14]. 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 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.

TABLE II.

Time in hours.	Percent of unesterified amino acid acidity.
0.00	74.9
0.50	55.6
1.00	39.9
2.00	47.3
4.00	42.5
6.00	32.6

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



after saponification, a method which would decolorize the entire solution before esterification without the addition of any inorganic salts or mineral acid 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 [16] 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 aluminium hydroxide formed. Instead of potassium alum and sodium carbonate, aluminium sulfate and barium hydroxide were used because they can easily be quantitatively removed from the solution. The hydrolyzate, after most of the hydrochloric acid had been removed by evaporation in vacuo, was made slightly alkaline with barium hydroxide. Ten grams of aluminium sulfate were then added for each hundred grams of protein used. The precipitate of aluminium hydroxide, barium sulfate and melanin was filtered off, leaving a clear yellow filtrate which was free from aluminium. To quantitatively remove the barium as the sulfate, it was found necessary to add a slight excess of the sulfuric acid and heat to boiling. All the barium was then precipitated as the sulfate, and the excess acid could be exactly removed by adding barium hydroxide. The filtrate after the removal of the barium as the sulfate was prepared for esterification as usual.

Three esterifications of mixtures of amino acids from,(I) 50 grams of casein,(2) 25 grams of vignin and (3) 50 grams of gel-

atin, were followed by the method modified as indicated above. The procedure in each case was the same in regard to time, temperature, etc. The temperature of the oil baths was kept sufficiently high, (about I30 degrees) so that the temperature of the esterifying mixture was between I00 and I05 degrees. In each case one liter of alcoholic hydrochloric acid was passed through at a constant rate for eight hours.

The usual samples were taken off at definite intervals and made up to 50 cc with 95 percent redistilled alcohol. A I0 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 dilute hydrochloric acid for a period of eighteen hours under a reflux condenser.[i]

[i] After saponifying by boiling this length of time, a finely divided black precipitate was formed. This was removed by filtering the sample after it was made up to a definite volume through a dry filter. No attempt was made to discover the nature of this precipitate.

The saponified sample was then evaporated on the steam bath to a small volume and made up to the original volume of twenty five cc. A I0 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 difference of the titrations before saponification will give the free(unesterified) amino acidity, while those after the saponification will give the total amino acid acidity in terms directly comparable to the first titrations. From these two values, expressed in cc of normal acid, the percent of unesterified amino acid acidity can be determined directly. The results of this series are given in table III in terms of normal acid,

although the standard solutions for the Sörensen titration were fifth normal and those for the Volhard titration tenth normal. Thus the error always accompanying the use of small volumes for titration was avoided.

TABLE III.

Experiment I, casein.

Before saponification.

Time in hours.	0.00[i]	0.50	I.50	3.50	5.50	8.00
Total acid.		3.965	3.336	2.586	4.I4I	3.434
Mineral acid.		2.2I3	2.562	I.995	3.520	2.870
Amino acid.		0.752	0.774	0.59I	0.62I	0.78I

After saponification.

Time in hours.	0.00[i]	0.50	I.50	3.50	5.50	8.00
Total acid.		4.86I	5.958	5.073	5.604	5.954
Mineral acid.		3.385	4.I47	3.494	3.832	3.543
Amino acid.		I.476	I.8II	I.579	I.772	2.4II
Percent of unesterified amino acid acidity.		50.9	42.6	37.5	35.6	24.2

[i] The sample taken off at the start gave impossible results and as there was not enough for a repetition of the analysis, the data was omitted from the table.

Experiment II vignin.

Before saponification.

Time in hours.	0.00	0.50	I.50	3.50	5.50	8.00
Total acid.	2.809	2.3II	3.369	2.277	3.792	2.I55
Mineral acid.	I.704	I.894	2.794	2.050	3.420	I.902
Amino acid.	I.I05	0.4I7	0.575	0.227	0.372	0.253

After saponification.

Time in hours	0.00	0.50	I.50	3.50	5.50	8.00
Total acid.	6.877	6.076	5.22I	3.046	6.346	3.2I3
Mineral acid.	5.690	4.702	3.302	I.866	4.002	I.934
Amino acid.	I.I87	I.374	I.9I9	I.I80	2.344	I.279
Percent of unesterified amino acid acidity.	93.I	30.4	29.9	I9.2	I5.8	I9.8

Experiment III, gelatin.

Before saponification.

Time in hours.	0.00	0.50	I.50	3.50	5.50	8.00
Total acid.	4.780	5.4I2	4.402	3.036	4.I66	3.49I
Mineral acid.	2.860	4.0I8	3.43I	2.474	3.508	3.0I8
Amino acid.	I.920	I.394	0.97I	0.562	0.658	0.473

After saponification.

Time in hours.	0.00	0.50	I.50	3.50	5.50	8.00
Total acid.	8.I44	9.I25	7.962	4.227	6.350	5.4I0
Mineral acid.	5.485	6.I82	4.695	2.464	3.798	3.I99
Amino acid.	2.659	2.943	3.267	I.763	2.552	2.2II
Percent of unesterified amino acid acidity.	72.2	45.8	28.7	3I.8	25.7	2I.4

No perceptible deepening of the color of the esterifying mixture was noticed. The slightly higher results obtained, for the extent of esterification, as compared with the values obtained in the previous experiments, may be attributed to the increased temperature and the larger proportional amount of alcohol passed through the esterifying mixture. 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 eight hours. It is probable that the period of esterification used in the work of Osborne and associates (six to eight hours) is not sufficiently long to secure maximal results.

The rate of esterification of the casein was 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, than 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 for such different proteins as casein, vignin and gelatin is significant. The fact that for each protein, the extent of esterification at the end of eight hours is approximately the same is also noteworthy, and would indicate that the unesterified amino acid acidity represents an approach to an equilibrium rather than to a definite unesterifiable fraction.

The accuracy of the above method of determining the rate and extent of the esterification of amino acids depends, primarily, on the accuracy of the determination of the unesterified amino acid acidity, which in turn depends on the stability of the amino acid esters under the conditions of the first Sörensen titration. It was found that in the absence of free alkali, amino acid esters in 95 percent alcohol were stable after eighteen hours standing.[i]

[i] Amino acid esters obtained from casein were placed in different strengths of alcohol. After eighteen hours standing, no saponification could be detected in the 95 and 80 percent alcoholic solutions; in the 60 percent alcohol, 6.4 percent of the amino acid esters was saponified, while in a water solution, 25 percent was saponified.

In view of the fact that the end point of the Sörensen titration corresponds to a weak alkalinity, some saponification was anticipated as the end point was approached. The first distinct color change, taken as the end point in this titration, disappeared in less than a minute, showing that there was an increase of acidity.

On testing this point with a solution of amino acid esters

in 95 percent alcohol, we found that saponification did occur. If the end point was approached as rapidly as possible, we found that, for samples taken off near the end of esterification, in which the concentration of esters was greatest, saponification may be limited to 5 to 7 percent of the esters present. We hope to reduce this saponification to a minimum and to determine, if possible an approximate correction factor,to be subtracted from the amount of alkali required to titrate the free amino acid acidity, which will embody both the correction factor for the saponification and the correction as determined by the regular blank control.

The results from tables I,II and III indicate that all of the amino acids are esterifiable and that an equilibrium point is reached in the reaction. So far as we are aware, no attempt has been made to determine whether or not the diamino acids are esterifiable, We were able to show that 70 percent of lysine could be esterified, even when the temperature of the bath was only 105 to 110 degrees; but we did not have a large enough sample to follow the rate of esterification. With a mixture of amino acids from casein, the esters of which had distilled over below 90 degrees under diminished pressure, and from which proline had been extracted by alcohol, we were only able to esterify 68 percent of the amino acid acidity, though in this case also the bath was raised only to 105 to 110 degrees. Thus,although all the amino acids present were esterifiable, an unesterified residue resulted. Both of these results indicate that an equilibrium point is reached, and that all the amino acids are esterifiable.

SUMMARY AND CONCLUSION.

The rate and extent of esterification of organic acids can best be followed by titrametric methods. In adapting such a method to the determination of the esterification of alpha amino acids, some means must be used to overcome the basisity of the amino group before the free amino acids can be titrated. The modification employed involves the use of the Sörensen formaldehyde 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 by condensation 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 water and hydrochloric acid, will give the total amino acid acidity. From these two results, the percent of unesterified amino acid can be readily calculated.

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

The method has been successfully applied to mixtures of amino acids from casein, vignin and gelatin, as well as individual amino acids, and shows that 75 to 85 percent of the amino acid acidity can be esterified in six to eight hours, or if a tentative correction for saponification is applied, 80 to 90 percent.

From the results obtained in this investigation, there appears to be no unesterifiable residue of amino acids. The reaction 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 alpha amino acids should be run, and in comparing the efficiency of different methods of esterification.

The method as above outlined can be made more accurate if a correction can be applied for the saponification of the esters occurring at or near the end point of the Sörensen titration.

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