XXXVIII. RAPID VOLUMETRIC METHODS FOR THE ESTIMATION OF AMINO-ACIDS, ORGANIC ACIDS AND ORGANIC BASES.

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(*Received April 2nd, 1920.*)

In the course of an investigation of the process of putrefaction, it became necessary to devise methods by which many estimations could be made of such products of enzyme or bacterial action as organic acids, amino-acids and organic bases. Existing methods such for instance as that for determining the organic acids volatile in steam, the estimation of amino-acids by Sörensen's formol titration method and the separation of organic bases by distillation were tried but found wanting either on account of the lengthy nature of the process or the inaccuracy of the results.

It was recognised that volumetric methods which would make possible the rapid and accurate estimation of mixtures of organic acids, amino-acids and organic bases would have such wide applications that it would be worth while devoting much time and trouble to the subject. The investigation has resulted in the discovery of a simple and accurate volumetric method which admits of general application to the analysis of such organic mixtures as result from the degradation of proteins or similar bodies by acids, enzymes or bacteria.

On treating a fluid in which putrefaction has been proceeding with nine volumes of alcohol as described in a previous paper [Foreman and Graham-Smith, 1917] proteins, albumoses, peptones and other substances whose presence might interfere with the estimation of simpler constituents are precipitated. The filtrate which is light yellowish brown in colour contains about 87 % alcohol. The clue which led up to the evolution of a successful method applicable to such alcoholic filtrates was found in an observation by Sutton [1900] that potash or soda will displace ammonia in equivalent quantities from ammonium salts in alcoholic solution at ordinary temperatures, whilst at the same time the ammonia forms no compound with phenolphthalein used as indicator. The acid in an ammonium salt can therefore be titrated with standard alkali in alcoholic solution. Sutton does not state the strength of alcohol necessary to secure this result. The alcoholic filtrates contain amines as well as ammonia together with about 13 % of water. It seemed reasonable

to assume that the amines might behave as ammonia towards phenolphthalein in alcoholic solution, in which case the bases would not interfere with the direct quantitative titration of the organic acids in the alcoholic filtrates provided the presence of a little water did not vitiate the results.

In order to provide a stock of standard material on which the method could be tested, the following procedure was adopted.

The body of a rabbit, including the skin and internal organs with their contents, was minced, and 2½ times the weight of water added in a Winchester quart bottle. The bottle was loosely plugged with cotton wool and kept at 25-30° for four weeks, in an inclined position so as to expose as great a surface as possible. The contents were lightly shaken once daily, the plug being removed during this operation. At the end of the period the fluid contained the products of advanced aerobic putrefaction. A portion was treated with nine volumes of 97 % alcohol, allowed to stand all night and filtered. The solution containing 87·3 % alcohol was stored in a stoppered bottle and will be referred to frequently in this paper as the "Alcoholic Extract." The following results were obtained on analysis of 50 cc. portions by the methods described in an earlier communication [Foreman and Graham-Smith, 1917].

Volatile bases	24·85 cc. $N/10$ acid
Formol titration of residual liquid (amino-acids)	2·1 cc. $N/10$ soda
Organic acids volatile in steam	19·7 cc. $N/10$ soda

The "Alcoholic Extract" was titrated under the differing conditions described in Table I, using phenolphthalein as indicator. The 97 % alcohol used was slightly acid to phenolphthalein, 100 cc. requiring 0·15 cc. $N/10$ alkali.

Table I. Preliminary Experiments with the "Alcoholic Extract."

Col. 1	2	3	4	5
Ref. No.	Reagents added to 10 cc. portions of "Alcoholic Extract" before titrating	Aq. $N/10$ soda required cc.	% alcohol in the mixture after titrating	Readings calculated to a basis of 50 cc. "Extract," after correcting for the original acidity of the alcohol
1	0	4·2	61	20·1
2	20 cc. 97 % alcohol	4·7	81	23·35
3	50 cc. „	4·7	88·5	23·13
4	50 cc. 97 % alcohol and 0·5 cc. ammonia (sp. gr. ·88)	4·7	87·8	23·13
5	50 cc. 97 % alcohol and 5 cc. aq. butyric acid solution (=7·6 cc. $N/10$ soda to phth.)	12·25 7·6 4·65	73·9	22·88
6	100 cc. 97 % alcohol and 5 cc. aq. butyric acid solution	12·4 7·6 4·8	83·0	23·25
7	50 cc. 97 % alcohol	4·75	88·5	23·38
8	To the titrated liquid of No. 7 added a neutralised mixture of 10 cc. formalin and 50 cc. 97 % alcohol	up to 4·85	84·7	23·88

The following observations were made during the carrying out of the first six experiments described in Table I. Reference will be made later to Exps. Nos. 7 and 8.

1. The light yellowish brown colour of the "Alcoholic Extract" practically disappeared on diluting with alcohol.

2. The endpoints were quite satisfactory.

3. On raising the percentage of alcohol from 61 to 81 the reading was increased, but between 81 and 88·5 % no further increase occurred.

4. The result was greater than previously obtained for the volatile organic acids in the same quantity of "Alcoholic Extract."

5. The presence of even a large amount of free ammonia did not affect the reading although the mixture contained 12 % of water.

6. The butyric acid added titrated quantitatively in the mixture.

The results showed that ammonia liberated by the alkali from the ammonium salts, which the "Alcoholic Extract" contained, did not affect the indicator when 81 to 88 % of alcohol was present. This inference was confirmed by obtaining quantitative results for the acid radicle on titrating a known quantity of pure ammonium chloride in 85 % alcohol.

As the "Alcoholic Extract" contained amines as well as ammonia, the behaviour of primary, secondary and tertiary amines towards phenolphthalein in alcoholic solutions was investigated. Known quantities of the pure hydrochlorides of these bases were titrated in 85 % alcoholic solution and found to require $N/10$ alkali quantitatively equivalent to the acid radicle in each case. The actual experiments will be found in a later section. These amines therefore resembled ammonia in forming no compound with phenolphthalein in the alcoholic solutions.

The effect of alcohol in different concentrations upon the titration value of the acid radicles when titrated in the presence of the free bases was then studied in detail. For this purpose 97 % alcohol was added in small quantities at a time to known quantities of standard aqueous solutions of ammonium salts and salts of primary, secondary and tertiary amines, and the liquids titrated with $N/10$ alkali after each addition using phenolphthalein as indicator. The total titration value increased as the content of alcohol in the solutions was augmented, reaching a maximum when 80 to 85 % alcohol was present. In the experimental section, a typical experiment of this nature carried out with trimethylamine hydrochloride is fully described. In this case the reading reached its highest point when the concentration of alcohol had been increased to 78 %.

Having determined that ammonia and amines form no compound with phenolphthalein in the presence of 85 % alcohol, the amount of $N/10$ alkali required to neutralise a certain volume of the "Alcoholic Extract" under this condition was regarded, at this stage of the investigation, as quantitatively equivalent to the organic acid radicles present. An attempt was therefore made to obtain a direct figure for the amino-acids in the "Alcoholic

Extract" by continuing the titration after adding a neutral alcoholic solution of formalin to the neutralised alcoholic solution. Experiments Nos. 7 and 8 of Table I, were therefore carried out. The additional $N/10$ soda required for neutralisation on adding the alcoholic formalin, however, was only 0·5 cc. per 50 cc. "Alcoholic Extract," whereas a reading of 2·1 cc. $N/10$ soda for the amino-acids in the same volume of "Alcoholic Extract" had been obtained previously by the formol titration of the residual liquid from the distillation of the volatile bases (p. 471). In attempting to find a reason for this deficiency, the behaviour of amino-acids was investigated under the same conditions. A 0·53 % aqueous solution of an amino-acid mixture, consisting of leucine and valine and containing 11·34 % N, was therefore treated and titrated as described in Table II.

Table II. Preliminary experiments with amino-acids.

Col. 1	2	3	4
Ref. No.	Treatment of the 0·53 % aq. solution of amino-acids before titrating	Aq. $N/10$ soda required (corrected) cc.	% alcohol in the mixture after titrating
9 (*a*)	5 cc. + 50 cc. 97 % alcohol	1·97	85·0
(*b*)	5 cc. + 100 cc. „	1·97	90·7
10	To the titrated liquid of Exp. 9 (*b*) added a neutralised mixture of 10 cc. formalin and 50 cc. 97 % alcohol	2·17	87·0

The result of Exp. No. 9 of the above table was most astonishing. Assuming each amino-acid to require one equivalent of $N/10$ soda for neutralisation, 5 cc. of the leucine-valine solution would theoretically require, calculated on the N content, 2·15 cc. $N/10$ soda. The figure actually obtained was 1·97 cc., which remained constant on increasing further the concentration of alcohol. These two amino-acids therefore titrated almost quantitatively as ordinary monobasic organic acids in 85 % alcohol using phenolphthalein as indicator, although in aqueous solution they are practically neutral. The result also showed that the reading obtained when the "Alcoholic Extract" was titrated in the presence of 81–88 % alcohol (see Exps. Nos. 2 and 3, Table I) represented amino-acids and organic acids together, and not the organic acids alone, as originally supposed.

The amino-acid solution resembled the "Alcoholic Extract" in requiring a small additional amount of $N/10$ alkali on adding alcoholic formalin to the neutralised alcoholic liquids.

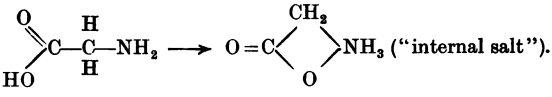
The surprising discovery that leucine and valine titrated in 85 % alcohol as if they were ordinary organic acids led to the supposition that this behaviour might prove common to amino-acids in general. If so, then the property might be utilised as a basis of a new titration method for the estimation of amino-acids. Other amino-acids were therefore submitted to investigation under the same conditions. The results of the experiments will be

given later. For convenience in presentation they may be summarised briefly as follows.

Phenylalanine, tyrosine, cystine, histidine, asparagine quantitatively required one equivalent of soda for neutralisation to phenolphthalein in the presence of 85 % alcohol, while the requirements of lysine and tryptophan were practically quantitative. Arginine was neutral. Glycine, alanine, leucine and valine gave a slight deficiency. The results obtained for aspartic and glutamic acid and proline however were considerably below the calculated value, with a corresponding lack of definiteness in the endpoints.

Hypothesis for explaining the behaviour of amino-acids in alcoholic solutions.

Assuming the neutrality of amino-acids in aqueous solutions to be due to the formation of "internal salts" as described by Mann [1906] an explanation of their behaviour in strong alcoholic solution on adding KOH seems possible. In the account given by Mann, glycocoll as a type is represented thus:



Walker [1904] believed that amino-acids in aqueous solution either form "internal salts" or unite in pairs in such a way that the basic radicle of one molecule links on to the acid radicle of another, the original trivalent N atom always becoming pentavalent whichever of these changes the amino-acid undergoes. Therefore the opinion was formed that the behaviour of amino-acids in alcoholic solutions on adding potash is comparable with that of an ammonium salt under the same conditions, the α-amino-group and ammonia being liberated from the "internal salt" and the ammonium salt respectively, and the potassium salts of the acid radicles formed. With its basic α-amino-group liberated from the "internal salt" the amino-acid can be regarded as a true substituted ammonia, which would be expected to behave similarly to ammonia and the simple amines in forming no compound with phenolphthalein in alcoholic solutions.

The effect of alcohol in different concentrations upon the basicity of the α-amino-group of amino-acids, was studied in the manner already described for investigating the basicity of ammonia and the amines (p. 453). The results obtained in two typical cases, viz.: histidine and arginine are given later (see Tables V and VI). Curves constructed from these data follow the same general contour as those obtained in a like manner for ammonium chloride and the hydrochlorides of the amines, the readings reaching the maximum at 80 to 85 % alcohol.

The abnormal behaviour shown by certain amino-acids titrated in alcoholic solution.

As already stated, certain amino-acids, more particularly aspartic acid, glutamic acid and proline, gave low results when titrated in their alcoholic solutions, although others behaved in accordance with the "internal salt" hypothesis (p. 455). Further work will be necessary before the deficiencies can be satisfactorily explained. Several possible explanations were considered. The following alternative suggestions both appear to receive support from the available evidence:

(1) That low titration values may be due to the union of a carboxyl group with alcohol.

(2) That condensation probably occurs in the alcoholic solutions.

At a much later stage, the discovery was made that the carboxyl groups of amino-acids will titrate in aqueous acetone in the same manner as they do in aqueous alcohol (see p. 457). Suggestion No. 1 is supported by the fact that aspartic acid, glutamic acid and proline, gave practically quantitative results when titrated in about 84 % acetone using phenolphthalein as indicator.

A comparison of the behaviour of aspartic acid and asparagine, on the other hand, seems to favour suggestion No. 2:

Aspartic acid	Asparagine
(*a*) $COOH$	$CONH_2$
CH_2	CH_2
$CHNH_2$	$CHNH_2$
(*b*) $COOH$	(*b*) $COOH$

Group (*a*) of aspartic acid titrated accurately in aqueous solution. On adding the stated amount of alcohol to this neutralised solution, group (*b*) required only about half the calculated equivalent of alkali to produce the faintest possible pink colour, the endpoint being very indefinite. The solution became faintly opalescent when the alcohol was added. These facts seem to support the suggestion that some kind of loose condensation, between group (*b*) of one aspartic molecule and the amino-group of another occurred in the alcoholic solution. Group (*b*) of asparagine however titrated quantitatively in alcoholic solution with a sharp endpoint. Therefore the phenomenon does not occur when no carboxyl group (*a*) is present. From this point of view it would appear that group (*a*) is involved in the condensation. Further investigation is proceeding.

Conditions under which the method becomes of general application.

The evolution of a successful general titration method for estimating amino-acids, based upon their behaviour in alcoholic solution, involved the necessity of overcoming the difficulty caused by certain amino-acids giving

low results. In endeavouring to achieve this purpose the significance of Exps. No. 8 of Table I and No. 10 of Table II was appreciated. Reference to these tables will show that the titration values of the "Alcoholic Extract" and the leucine-valine mixture, obtained in alcoholic solution, were slightly increased on adding alcoholic formalin, indicating that formaldehyde might be capable of disrupting any loose combination or condensation. A test was therefore made with glutamic acid. On adding alcoholic formalin to the neutralised alcoholic solution, sufficient further alkali was required to bring the total titration value up to the calculated figure. Subsequently every amino-acid giving a low result when titrated in alcohol alone was found to titrate quantitatively on adding alcoholic formalin. An accurate general method was therefore possible, provided those amino-acids which behaved normally in alcohol alone continued to do so in the presence of formaldehyde.

Histidine and tryptophan gave good results when titrated in alcohol alone. As these amino-acids contain an imino-grouping, methylene derivatives capable of neutralising acid when in aqueous solution are formed by the action of formaldehyde. Therefore it was necessary to show that such bases, which presumably would be formed on adding alcoholic formalin to the neutralised alcoholic solution of a complete amino-acid mixture, would be inactive to phenolphthalein, in which case they would not interfere in the general method. A study of the basicity of the basic methylene derivative of diethylamine, obtained by the action of formaldehyde upon the hydrochloride of this amine, showed that bases of this type, in common with ammonia, the amines and the freed amino-groups of amino-acids, do not form ionisable compounds with phenolphthalein if sufficient alcohol is present. The basic methylene derivative ceased to react with the indicator on increasing the concentration of alcohol to 70–75 %. The formation of basic methylene compounds of such amino-acids as histidine and tryptophan would therefore not be likely to vitiate the titration results. This statement was confirmed in actual experiments with these two amino-acids.

Low readings in alcohol raised on adding acetone.

Preliminary experiments have shown that acetone has the same effect as alcohol in submerging the basicity of the basic groups of amino-acids towards phenolphthalein. Aspartic acid, glutamic acid and proline, which gave low titration values in alcohol alone, titrated quantitatively in 80–85 % acetone. The behaviour of the other amino-acids has not yet been investigated. As an important differentiation of amino-acids in a mixture may be obtained by first titrating in 85 % alcohol and then continuing the titration after adding alcoholic formalin, the effect of adding acetone as an alternative to alcoholic formalin was tried in the case of aspartic and glutamic acids. These experiments, described later, showed that acetone was equally effective in raising the titration value to the quantitative figure. A thorough investigation of the effect of acetone is proceeding.

Description of the general method for estimating the carboxyl-groups of amino-acids.

Preparation of solution. A known weight of the amino-acid, or its suitable salt, or of an amino-acid mixture is dissolved in CO_2-free water and made up to a known volume so that the resulting solution is approximately $N/10$ in strength in relation to the carboxyl-groups. In cases such as tyrosine and cystine where the amino-acid is insoluble in water or soluble in too large a volume of water, $N/10$ HCl or $N/10$ soda or other appropriate means must be used for bringing them into solution so that no separation occurs on adding alcohol. When only a very small quantity of the amino-acid is available it may be weighed accurately into a suitable vessel dissolved in the desired amount of water and the whole of the solution used.

Stage 1. *Titration in water.* A 5 cc. or 10 cc. portion of the solution is titrated with aq. $N/10$ soda to phenolphthalein (soda standardised to phenolphthalein).

Stage 2. *Titration in alcohol.* 5 cc. or 10 cc. of the original solution is transferred to a conical flask of about 250 cc. capacity, 10 volumes of 97 % alcohol (rect. sp.) and three drops of phenolphthalein solution added, and the mixture titrated in daylight over a white plate with $N/10$ alcoholic potash (standardised to phenolphthalein) until a light pink colour is produced (p. 460). The endpoint is usually obtained quite sharply on adding the last one or two drops of standard alkali (two drops = 0·05 cc.). A correction is made for the original acidity of the alcohol.

Stage 3. *Titration in alcoholic formaldehyde.* After obtaining the reading in Stage 2, the same liquid is used for Stage 3. 12·5 cc. of aqueous formaldehyde solution prepared by diluting colourless formalin, with two volumes of distilled water, and neutralising the mixture to phenolphthalein, is added for each 50 cc. alcohol used in Stage 2, and the titration continued to the same endpoint as before. A mixture of neutralised diluted formalin and alcohol in the same proportions as they have been used is then titrated and the result obtained on completing Stage 3 is corrected accordingly. The acidity of rectified spirit is very slightly increased on adding the neutral formaldehyde solution.

The result obtained in Stage 1 gives useful information when dealing with the dibasic amino-acids, arginine, and salts of amino-acids (see p. 467).

The Stage 2 titration value includes the reading obtained in Stage 1. Several amino-acids are correctly estimated in Stage 2.

The increase in titration value obtained in Stage 3 gives an idea of the character of the amino-acid, and when dealing with an amino-acid mixture affords an indication of the amount of dibasic amino-acids and proline present. The carboxyl-groups of all the amino-acids contained in an amino-acid mixture, except that of arginine, are estimated by the total titration value obtained on completing Stage 3.

To avoid dilution of the alcohol, the use of $N/10$ alcoholic potash instead of $N/10$ soda was adopted. Since four drops of this reagent from an ordinary standard burette measure only 0·1 cc., a further advantage is gained in titrating. The alcoholic potash should be made from absolute alcohol and be free from carbonate. It should be as nearly colourless as possible and carefully guarded against evaporation.

The experiment with the basic methylene derivative of diethylamine showed that acid radicles can be titrated in the presence of such bases, if the concentration of alcohol is not less than 70 to 75 %. The addition of the stated amount of diluted aqueous formalin instead of alcoholic formalin was adopted at this stage, as it was found that sufficient formaldehyde was thus provided and at the same time the concentration of alcohol was not made lower than shown necessary in the experiment referred to. This slight modification proved more convenient.

The titrations should be done promptly in order to minimise any possible effect of CO_2 absorbed from the air.

Wider application of the method.

It will be evident from a study of the experimental results that the method outlined above is capable of much wider application in the following ways.

1. Aqueous solutions containing salts of ammonia, primary, secondary and tertiary amines and allied bases, either separately or as mixtures, can be investigated quantitatively, by means of Stage 2 of the method. The titration value thus obtained would represent the exact equivalent of the acid radicles present. If the original solution contained free acids as well as these salts, the result would include the equivalent of the free acids. The presence of free organic bases however would not influence the titration value.

2. In the case of mixtures of amino-acids and organic bases, the carboxyl-groups of the amino-acids, not including that of arginine, would be correctly estimated by the total titration value obtained on completing Stage 3 of the method. At the same time, the difference between the total titration value and the result of Stage 2, would give an indication of the content of dibasic amino-acids and proline, if present.

3. Animal products for example, muscle and organ extracts, faeces, urine, milk, blood, etc., vegetable extracts, and fluids containing the products of bacterial growth, may be investigated by the general method after treating in the manner described for preparing the "Alcoholic Extract" (p. 452). In many of these fluids, a mixture of organic acids, amino-acids, and organic bases occurs. The total titration value obtained on completing Stage 3 of the method would represent the total acid radicles present including the carboxyl-groups of the amino-acids, except that of arginine. The increase in titration value obtained by proceeding from Stage 2 and completing Stage 3 would approximately be proportional to the amount of dibasic amino-acids and proline present.

Experimental Section.

The experimental results illustrating the development of the new general method for estimating amino-acids will be given, as far as possible in the same order as they have been referred to in the foregoing section. Since Sörensen's formol titration method had been found to give very deficient results when titrating the acid radicles in salts of secondary amines, and to be unreliable in many cases when used for estimating amino-acids, each solution made up for the purpose of testing the new general method was also submitted to formol titration so that the results obtained by the two methods could be compared. Much difficulty was experienced in the formol titrations owing to the indefinite character of the endpoints (see p. 467). The light pink colour obtained sharply in titrating solutions by the new method was adopted as a standard and attempts were made throughout the formol titrations to obtain a colour of the same intensity. The results given in this section for all the titrations in alcohol or in alcoholic formaldehyde have been corrected for the original acidity of the alcohol or alcoholic formaldehyde.

The effect of alcohol upon the basicity to phenolphthalein of ammonia, primary, secondary and tertiary amines.

Standard aqueous solutions of the pure hydrochlorides of these bases, approximately $N/10$ in strength, were made up and 5 cc. or 10 cc. portions from each solution were titrated as described in Table III.

Table III.

Col. 1	2	3	4	5
Ref. No.	Substance	Formol titration $N/10$ alkali cc.	Titration in 85–90 % alcohol $N/10$ alkali cc.	Theoretical value of the acid radicle in the portion taken $N/10$ alkali cc.
1	Ammonium chloride	9·3	9·35	9·35
2	Ammonium sulphate	—	9·95	10·0
3	Monoethylamine hydrochloride	5·9	6·1	6·13
4	Dimethylamine hydrochloride	3·1 to 3·9	6·8	6·72
5	Diethylamine hydrochloride	0·85 to 1·0	9·02	9·13
6	Trimethylamine hydrochloride	0	4·72	4·6

Remarks. On adding the alcohol to the aqueous ammonium sulphate the salt separated. It was found that the separation could be prevented by previously dissolving 10 % glycerol in the alcohol. This mixture was neutralised before use. In the case of Nos. 4, 5 and 6 in Col. 4 the endpoints were especially sharp. The endpoints of Nos. 4 and 5 in Col. 3 were very indefinite. The figure for No. 6 in Col. 5 was determined by analysis.

Discussion. The results of the titrations in alcohol were practically quantitative in all cases.

Formol titrations of the salts of secondary amines showed that the basic methylene compounds were capable of neutralising the acid radicles to a large extent. The acid radicle of the tertiary amine salt possessed no formol titration value. It is interesting to note that methylene derivatives of secondary amines, *e.g.* $(C_2H_5)_2.N.CH_2.N.(C_2H_5)_2$ are tertiary amines, not sufficiently strong however to titrate quantitatively to phenolphthalein. This fact limits the usefulness of the formol titration method.

The effect of alcohol in different concentrations upon the basicity of trimethylamine.

5 cc. of the standard aqueous solution of the hydrochloride used in Exp. No. 6 of Table III was diluted with 5 cc. water. 97 % alcohol was added in small quantities at a time, and the liquid titrated with $N/10$ alcoholic potash after each addition. As too much alcohol had been added in the third increment a further 10 cc. water was added after taking the third reading. The readings were corrected for the original acidity of the alcohol, and the percentage of alcohol in the solution at the end of each titration was calculated.

The results given in Table IV are shown graphically in Fig. 1.

Table IV.

97 % alcohol cc.	none	1·0	3·5	13·5	23·5	33·5	43·5	53·5	63·5	78·5	103·5	153·5
Readings (corr.) $N/10$ alcoholic KOH cc.	0·02	0·07	0·5	2·78	4·19	4·5	4·59	4·67	4·73	4·78	4·78	4·78
% alcohol	0	9·4	28·0	43·8	56·7	63·7	68·7	72·4	75·2	78·3	82·1	86·2

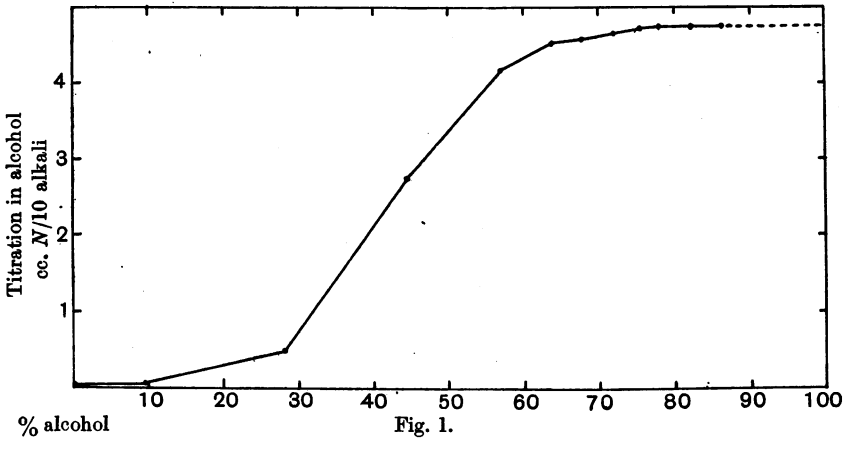


Fig. 1.

Discussion. The reading reached its highest point at 75–78 % alcohol, showing that at this concentration the free base is inactive to phenolphthalein. Similar results have been obtained on titrating ammonium chloride and the hydrochlorides of the other amines in the same way.

The effect of alcohol upon the basicity of the basic groups of amino-acids towards phenolphthalein.

The results of the experiments will be found in Table X, p. 466.

The effect of alcohol in different concentrations upon the basicity of the α-amino-group of amino-acids.

Experiment 1. 0·524 g. histidine monohydrochloride ($C_6H_9O_2N_3$, $HCl.H_2O$) was dissolved in water and made up to 50 cc. 10 cc. of the solution were neutralised to phenolphthalein with aq. $N/10$ soda. 5·1 cc. were required. Successive quantities of 97 % alcohol were then added, and the liquid titrated after each addition, exactly as described in investigating the basicity of trimethylamine. The results are given in Table V.

Table V.

97 % alcohol added cc.		1·0	2·0	3·0	4·0	5·0	10·0	15·0	20·0	30·0	40·0	50·0	75·0	100·0	150·0
Readings (corr.) $N/10$ alcoholic potash	cc.	0·05	0·2	0·5	1·2	1·7	3·38	3·88	4·22	4·63	4·79	4·88	4·97	5·1	5·12
% alcohol		6·5	12·7	19·0	25·0	30·0	46·0	54·3	60·0	67·8	72·8	76·3	81·8	85·0	88·5

Remarks. The endpoints were not so distinct in the first five cases.

Discussion. In making up the solution the molecular weight of the salt was regarded as representing two equivalents of acid, one for the chloride radicle and the other for the carboxyl-group. The solution was made exactly $N/10$ on this basis. The chloride radicle in the pure salt titrated practically quantitatively in aqueous solution. According to Plimmer [1915] histidine is alkaline in reaction. The result does not support this statement so far as phenolphthalein is concerned.

The results show that the α-amino-group of histidine, liberated by the potash, behaved as ammonia and the amines to phenolphthalein in alcoholic solutions.

Experiment 2. 0·375 g. very pure arginine dinitrate ($C_6H_{14}O_2N_4$, $2HNO_3$) was dissolved in water and made up to 50 cc. The solution was thus exactly $N/20$ in relation to the $2HNO_3$. 10 cc. were neutralised with aq. $N/10$ soda to phenolphthalein. 2·5 cc. were required. 7·5 cc. water were then added and the experiment continued exactly as described in the case of the histidine hydrochloride. The results are given in Table VI.

Table VI.

97 % alcohol cc.		1	1½	2	3	4	5	10	15	20	30	40	50	100	150	200
Readings (corr.) $N/10$ alcoholic potash	cc.	0·02	0·07	0·15	0·25	0·54	0·69	1·64	2·08	2·27	2·45	2·47	2·5	2·55	2·55	2·55
% alcohol		4·8	7·1	9·4	13·6	18·0	21·6	35·9	44·9	51·3	60·2	66·1	70·4	81·3	85·8	88·3

Discussion. As explained later (p. 468), on adding alcohol to the neutralised aqueous solution, the α-amino-group of arginine was liberated by the alkali from its internal combination with the carboxyl-group. The basicity of the free α-amino-group to phenolphthalein was completely submerged when the concentration of alcohol was increased to a sufficient extent.

The effect of different concentrations of alcohol upon the basicity to phenolphthalein of the basic methylene derivative of diethylamine.

The basic methylene derivative was made by adding 30 cc. neutralised diluted aqueous formalin (2 vol. water to 1 vol.) to 10 cc. of the standard aqueous solution of diethylamine hydrochloride used in the experiment No. 5 of Table III. The mixture was neutralised with $N/10$ soda to phenolphthalein, 1·0 cc. being required. Alcohol was then added in small quantities at a time and the experiment continued exactly as described in the case of the salts of trimethylamine, histidine and arginine. The results are given in Table VII.

Table VII.

97 % alcohol cc.	none	1·0	2	3	5	10	20	33	40	50	75	100	150	175
Readings (corr.) $N/10$ alcoholic potash cc.	1·05	1·2	1·5	2·39	4·09	6·38	7·96	8·53	8·77	8·85	9·05	9·15	9·2	9·22
% alcohol	2·56	5·1	7·9	11·7	18·2	28·5	40·3	49·8	53·7	58·1	66·0	71·2	77·7	79·9

Discussion. The acid radicle derived from the original amine salt titrated quantitatively when the concentration of alcohol was increased to 71 %. The basic methylene derivative, in common with ammonia, the amines and the free α-amino-group of amino-acids forms no ionisable compound with phenolphthalein when sufficient alcohol is present.

The low values given by certain amino-acids when titrated in alcohol are raised on adding formaldehyde to the neutralised alcoholic solutions.

The experimental evidence will be given in Table X, p. 466.

Effect of acetone upon the basicity of the basic groups of amino-acids towards phenolphthalein.

A thorough investigation of this question is proceeding. Up to the present only four of the amino-acids have been tested. Portions of standard aqueous solutions were first titrated with aq. $N/10$ soda to phenolphthalein. Acetone was then added, and the titrations continued. The results are given in Table VIII. Corrections have been made for the original acidity of the acetone.

Remarks. Glycine separates from aqueous acetone when the concentration of acetone reaches about 73 %. In Exp. No. 1, the glycine redissolved as the titration proceeded. Only a faint opalescence resulted on adding the acetone in Nos. 3 and 5. The results of Nos. 4 and 6 were obtained by continuing the titration after adding further acetone to the neutral liquids from Nos. 3

and 5 respectively. A small separation which remained in suspension was produced by the additional acetone in each case. This probably consisted of the sodium salts, as apparently the titration of the carboxyl-group was not disturbed.

In the case of proline about 0·2 cc. further soda was required on adding more acetone.

Table VIII. Preliminary experiments.

Ref. No.	Substance	Titration in aqueous solutions aq. $N/10$ soda cc.	Further aq. $N/10$ soda required on adding acetone cc.	Total titration value cc.	% acetone at the end of titration	Theoretical total titration value of portion taken cc.
1	Glycine	0·05	5·2	5·25	83·3	5·0
2	Proline	0·15	5·1	5·25	76·3	5·28
3	Aspartic acid	3·75	3·7	7·45	75·1	7·5
4	„ „	3·75	3·8	7·55	84·8	7·5
5	Glutamic acid hydrochloride	9·9	4·75	14·65	71·8	15·0
6	„ „	9·9	5·0	14·9	83·4	15·0

Discussion. In the case of glutamic acid a higher concentration of acetone than 71·8 % was required. The results show that the carboxyl-groups of these amino-acids titrate practically quantitatively if sufficient acetone is present. The general effect of acetone resembles that of alcohol.

The low titration values given by certain amino-acids in alcohol are raised on adding acetone to the neutralised alcoholic solutions.

Up to the present only aspartic and glutamic acids have been investigated. Portions of standard aqueous solutions were treated with the requisite amount of alcohol and titrated with aq. $N/10$ soda to phenolphthalein. Acetone was then added, and the titrations continued. The results are given in Table IX.

Table IX. Preliminary experiments.

Col. 1	2	3	4	5
Substance	Titration in alcohol, aq. $N/10$ soda cc.	Total titration value on adding acetone, $N/10$ soda cc.	% acetone at the end of titration	Theoretical total titration value of the portion taken cc.
Aspartic acid	6·3	7·6	28·1	7·5
Glutamic acid hydrochloride	12·65	14·85	45·0	15·0

In these two experiments the endpoint colour of the indicator showed a tendency to fade when obtaining the figures of col. 3. This phase extended over a range of about 0·2 cc. The fading was probably due to the effect of CO_2 absorbed from the air. This point needs further investigation.

The results show that to whatever cause the low titration values in alcohol are attributed (see p. 456) the addition of acetone or formaldehyde brings

about a disturbance of the equilibrium so that the total titration values are practically quantitative. It is evident that the action of these two reagents requires thorough investigation.

Estimation of carboxyl-groups of amino-acids by the new method.

Accurate $N/10$ solutions of the amino-acids were made up, the quantity necessary for the purpose in each case being calculated on the basis of the number of carboxyl-groups in the molecule. In the investigation of the amino-acid salts, the acid radicles as well as the carboxyl-groups were taken into account in the calculations. When dealing with mixtures, the calculation was based on the nitrogen content. A description of the specimens used and the modified procedure adopted for amino-acids of low solubility are given below:

1. Glycine pure.
2. Leucine 10·8 % N.
3. Phenylalanine, very pure, prepared from the pure hydrochloride.
4. Tyrosine, very pure. Three times crystallised.

0·0906 g. was dissolved in 7·5 cc. aq. $N/10$ HCl by dipping the vessel into hot water for a few seconds. After cooling, the requisite amount of alcohol was added, and the titration done promptly. Allowance was afterwards made for the acid used.

5. Cystine, very pure. Colourless hexagonal plates under microscope.

0·0601 g. was dissolved in 6 cc. cold aq. $N/10$ alkali. The right amount of alcohol was then added and the excess of alkali determined by titrating with $N/10$ HCl.

6. Tryptophan, very pure. Colourless glistening platelets.

0·102 g. was dissolved in 5 cc. water at about 85°, cooled, the alcohol added and titrated promptly.

7. Proline. Completely soluble in cold absolute alcohol.

Crystallised in desiccator. M.P. 216°. Contained only traces of impurity represented by a very small content of amino-nitrogen.

8. Asparagine. Large crystals.
9. Aspartic acid. Contained traces of insoluble impurity.

0·3328 g. dissolved in about 40 cc. hot water, cooled and made up to 50 cc.

10. Glutamic acid hydrochloride. Twice crystallised.
11. Histidine monohydrochloride. $C_6H_9O_2N_3$, $HCl.H_2O$. Very pure. M.P. 255–256°.
12. Arginine dinitrate $C_6H_{14}O_2N_4$, $2HNO_3$. Very pure.

Specimen completely freed from all traces of free HNO_3 by standing over potash in a desiccator for a long time, then thoroughly dried over $CaCl_2$. M.P. 145° (uncorr.) decomposing 150–160°.

13. Lysine picrate, pure recrystallised.

0·0938 g. dissolved in 10 cc. water by dipping vessel into hot water for a few seconds. Alcohol then added and the mixture titrated at once.

The results are given in Table X.

Table X.

Ref. No.	Substance	Col. 1	2	3	4	5	6
				New General Method			
			Titration in aqueous HCHO. Solutions not neutralised previously. aq *N*/10 soda cc.	Stage 1 Titration in water aq *N*/10 soda cc.	Stage 2 Titration in alcohol *N*/10 alc. potash cc.	Stage 3 Titration in alcoholic formaldehyde *N*/10 alc. potash cc.	Theoretical total titration value of the portion taken *N*/10 alkali cc.
	Amino-acids:						
1	Glycine		5·05	0·05	4·82	5·15	5·0
2	Leucine		4·3	—	4·7	5·1	5·05
3	Phenylalanine		4·65	—	5·0	5·02	5·0
4	Tyrosine		4·3	—	5·0	5·12	5·0
5	Cystine		4·87	—	5·0	5·05	5·0
6	Tryptophan		—	—	4·92	5·15	5·0
7	Proline		0·82	0·07	2·07	2·6	2·64
8	Asparagine		5·0	0·55	5·02	5·3	5·0
9	Aspartic acid		4·3	2·47	4·2	4·95	5·0
	Salts of Amino-acids:						
10	Glutamic acid hydrochloride		6·9	4·9	6·4	7·45	7·5
11	Histidine monohydrochloride		3·75	2·55	5·0	5·05	5·0
12	Arginine dinitrate		4·9	2·5	5·05	5·15	5·0
13	Lysine picrate		4·5	—	4·9	5·05	5·0
	Mixtures of Amino-acids:						
14	Glycine, alanine, leucines and valine		6·3	0·2	7·05	7·5	7·5
15	The monobasic amino-acids from the hydrolysis of caseinogen		2·32	—	2·75	3·0	?
16	Mixture of the same quantity of the solution used for No. 10 with half the quantity used for No. 15		—	—	8·12	9·05	9·0

Remarks. The titration values obtained in Stage 2 include those obtained in Stage 1.

In obtaining the results for Nos. 7, 9 and 10 in Stage 2, the faintest possible pink colour appeared at 1·75 cc., 3·8 cc. and 6·2 cc. respectively. No. 10 gave 6·6 cc. in Stage 2 when titrated hot.

The titrations of lysine picrate were difficult owing to the light brownish yellow colour of the solutions. Several readings were taken in the presence of an independent observer in the following manner: after each reading the liquid was made acid by adding a known quantity of *N*/10 HCl and again titrated with *N*/10 alkali. After allowing for the acid added in each case, the following results were obtained in succession:

Stage 2: 4·9, 4·95.

Stage 3: 5·05, 5·1, 5·05, 5·05. Colour too pronounced at 5·25 cc. No other pure lysine salt was available.

Picric acid was found to titrate quantitatively as a monobasic acid in

both Stages 1 and 2. Potassium picrate separated from the alcoholic solution on standing.

The mixture used in No. 15 consisted of the amino-acids recovered after removing alcohol-insoluble calcium salts from the hydrolytic products of caseinogen.

The results given in Table X may be summarised as follows:

Formol titration method.

Glycine and asparagine	quantitative results.
Cystine	only 2 % low.
Arginine (net result)	practically neutral.

In all other cases considerable deficiencies were shown.

New method.

Stage 1. Quantitative results were obtained for the following carboxyl-groups and acid radicles:

Dibasic amino-acids	One of the carboxyl-groups.
Histidine monohydrochloride ...	The chloride radicle.
Arginine dinitrate	One of the two nitrate radicles.

Stage 2. Phenylalanine, tyrosine, cystine, histidine, and asparagine gave quantitative titration values:

Tryptophan and lysine, practically quantitative.

Arginine (net result), quantitatively neutral.

Glycine, 3·5 % low.

Leucine and the mixture of glycine, alanine, leucines and valine, 6–7 % low.

Proline, 20–30 % low.

Aspartic and glutamic acids, 30–45 % low in relation to the value of one carboxyl-group.

Stage 3. Practically quantitative results in all cases.

Discussion. Formol titration method. In carrying out the titrations in aqueous formaldehyde, 30 cc. diluted formalin (1 vol. to 2 vols. water) neutralised immediately before use were used in each experiment. It was found necessary to dilute the formalin before it could be neutralised precisely to phenolphthalein. In a few cases the titration values were slightly increased on adding further neutral formaldehyde. When this happened the excess was made greater until the reading reached a maximum. No. 14 gave the highest increase obtained in this way, viz. by 0·35 to 6·65 cc. Tyrosine gave an increase by 0·2 cc. to 4·5 cc. In the other cases (including glycine) the increase was only about 0·1 cc.

Phenolphthalein was found to indicate very indefinitely in the formol titrations. Sörensen [1907] appears to have encountered the same difficulty, as he advocated the addition of 0·2 *N* alkali until a deep red colour developed, to be matched with a deep red colour prepared under known conditions in a control solution. The formol results given in col. 2 of Table X were obtained by titrating, until beyond all doubt the solutions were light pink in colour

(p. 460). If the opinion be held that the solutions are still acid, although a light pink colour has developed, then the indicator is unsuitable, and the results are unsatisfactory.

Abnormalities in the formol titration method are fully discussed by Jodidi [1918], who states that accurate results are given when the amino-acid molecule contains "amino- and carboxyl-groups *only*," and that if other basic groups, such as the imino-group, are present, the method is unsuitable. The results obtained in this present investigation confirm the observations of Jodidi save in regard to the monobasic monamino-acids, which all gave low titration values with the exception of glycine. Jodidi attributes the adverse influence of the imino-group to the effect of the basic methylene derivatives produced by the action of formaldehyde.

The new method. With the exception of aspartic acid, glutamic acid and proline, the endpoints of the titrations in alcohol (Stage 2) were quite satisfactory. Especially was this the case with those amino-acids which gave quantitative results. All the titrations in Stage 3 of the method gave satisfactory endpoints. The sharpness with which the light pink endpoint appears is well shown on titrating a little pure phenylalanine or trimethylamine hydrochloride in 90 % alcohol. If desired the endpoint so obtained may be used as a standard.

Arginine. In making up the standard solution of arginine dinitrate, the molecular weight of the salt was regarded as representing two equivalents in relation to the $2HNO_3$. The solution was made exactly $N/20$ on this basis. 10 cc. required 2·5 cc. $N/10$ alkali when titrated in aqueous solution, and double this quantity when titrated in alcoholic solution. The salt therefore behaved as a monobasic acid in water and as a dibasic acid in alcohol. This behaviour can only be explained by assuming that the guanidine nucleus is combined with one HNO_3 in the aqueous solution of the dinitrate, and that the other HNO_3 combines with an equivalent of alkali during the titration. In the neutralised aqueous solution therefore the α-amino-group and the carboxyl-group form an "internal salt." On adding the requisite amount of alcohol to the neutralised aqueous solution, the alkali equivalent of the carboxyl-group is required as in the case of other amino-acids. Consequently, the guanidine nucleus reacts as a univalent base to phenolphthalein in both aqueous and alcoholic solutions. This is the only nitrogenous base or basic group of those investigated up to the present, possessing the power of reacting with the indicator in alcoholic solution. Other guanidine derivatives and guanidine have been investigated under the same conditions. The results show that the reaction to phenolphthalein in alcoholic solution is a common property when the molecule contains a NH grouping attached to a carbon atom by a double bond. An account of this investigation will be given in another communication.

The neutrality of arginine to phenolphthalein in alcoholic solution distinguishes it from other amino-acids. The utilisation of this character as the

basis of a new method for estimating arginine in the hydrolytic products of proteins is under investigation.

Since the completion of the experimental work described in this paper, I found that Victor Birckner [June, 1919], when titrating grain extracts, noted that the acidity to phenolphthalein increased on adding alcohol to the aqueous solutions. The phenomenon was attributed to amino-compounds in the extracts. Birckner's main conclusions were summarised by him as follows: "Amino-acids which in aqueous solution are nearly neutral to phenolphthalein react distinctly acid in the presence of alcohol. This fact should be taken into account when making acidimetric titrations in alcoholic liquids containing amino-compounds such as various animal and vegetable extracts." The amino-acids Birckner tested were those which do not give quantitative results even in much higher concentrations of alcohol than he employed.

The fact that amino-acids become acid in alcoholic solutions was discovered quite independently and by an entirely different route.

Wider application of the method.

Further evidence of the quantitative character of the estimation of total acid radicles including the carboxyl-groups of amino-acids, by the new method, was obtained by titrating known mixtures of glycine and butyric acid. The results corresponded with the amounts known to be present.

The "Alcoholic Extract" which had been stored for several months, since the experiments of Table I were carried out, was again investigated by the method. 50 cc. required 23·37 cc. $N/10$ alkali in Stage 2 and 23·75 cc. in Stage 3. By comparing these figures with those given in Table I it will be noted that such preparations as the "Alcoholic Extract" can be preserved for a considerable time without incurring appreciable loss or change.

A definite quantity of the solution of the mixture of amino-acids used in Exp. No. 15 of Table X was added to a portion of the "Alcoholic Extract." The titration values obtained in Stages 2 and 3 were found to include the effect of the additional carboxyl-groups quantitatively.

It is evident that by the use of this method, data may be accumulated quickly in the investigation of many important problems, amongst which the following may be mentioned.

(1) The order of breakdown of the constituents of animal matter by the organisms of putrefaction, with a view to obtaining clues as to the best means of detecting the early stages of putrefaction in meat or fish, so that the quality of these materials may be gauged.

(2) The breakdown, separately in artificial media, of proteins, fats and carbohydrates by enzymes and bacteria. The action of pathogenic or other organisms in pure culture upon various sugars and amino-acids. Decarboxylation of amino-acids.

(3) Rapid estimations of organic acids in faeces with the object of throwing

light upon the work of organisms in the intestines as affected by unsuitability of rations or disproportion in the essential food constituents.

(4) Comparison of foods by experiments designed to imitate digestion.

(5) Investigation of extracts of agricultural products such as silage.

(6) Rate of hydrolysis of proteins, and the investigation of the order in which the amino-acids are split off.

(7) Investigation of the purity of specimens of amino-acids or their salts. It will be noted that the amino-acids are not destroyed during their investigation by means of Stage 2 of the method, and can therefore be recovered. When only small amounts of material are available this fact is of obvious importance.

Further investigations are proceeding in these directions.

The rapid estimations of volatile bases and amino-acids.

In the older method for estimating volatile bases and amino-acids in "Alcoholic Extracts" [Foreman and Graham-Smith, 1917], it was necessary to distil the bases at a low temperature in order to avoid the decomposing effect of the excess of alkali upon the amino-acids. A considerable amount of time and attention was therefore required in carrying out the distillations.

The total titration value of an aliquot portion of the "Alcoholic Extract," determined by completing Stage 3 of the new method, affords an exact measure of the amount of alkali necessary for liberating the bases from their salts. If no more than this amount be added a rapid method of distillation at a higher temperature can be adopted because decomposition of amino-acids is reduced to a minimum. The residual liquid becomes aqueous as the alcohol is removed and the alkali salts of the amino-acids hydrolyse, so that an amount of free alkali equivalent to the amino-acids is found in the aqueous solution.

An improvement in the titration of the volatile bases distillate is rendered possible by finding that a sharp endpoint can be obtained in all concentrations of alcohol when alizarin is used as an indicator. The endpoint is so sharp that one drop $N/10$ alkali in excess produces a very decided colour change. As the distillates can be titrated without adding water, a further advantage is gained by using alizarin instead of methyl orange.

The method used was as follows:

50 cc. "Alcoholic Extract" was placed in the flask of an ordinary steam distilling apparatus and the correct amount of $N/10$ alcoholic potash added. This amount had been ascertained in another portion by completing Stage 3 of the new method. A rapid current of steam derived from distilled water which had been freed from CO_2 by prolonged boiling, was passed through the solution until a foam nearly filling the flask developed. Air was admitted into the flask in which the steam was generated in time to prevent any of the foam from passing into the condenser. The foam usually appeared in four to five minutes. The steam inlet tube was then raised above the surface of the

liquid and the apparatus swept by a further current of steam for one minute. The bases with the alcohol were carried from the condenser to the receiver by a tube which dipped into a known quantity of $N/10$ acid. The excess of acid in the alcoholic contents of the receiver was determined by titrating with $N/10$ soda (standardised to alizarin) using alizarin as indicator. The amount of acid equivalent to the volatile bases was estimated by difference. The distillates need no dilution with water before titrating. The indicator can be added in the form of a weak aqueous emulsion, or in 0·5 % alcoholic solution.

The flask containing the residual liquid, which appeared to be quite free from alcohol, was cooled under the tap, the steam inlet tube washed with water and removed, and the solution titrated without delay with $N/10$ acid to phenolphthalein. Even if a very small amount of alcohol were present, its influence upon the titration value in the aqueous solution would be inappreciable (see Tables V and VI). The content of free alkali in the residual liquid represents approximately the difference between the total titration value of the carboxyl-groups of the amino-acids and the effect of those groups which titrate to phenolphthalein in aqueous solution.

By this method, the bases from three successive 50 cc. portions of the "Alcoholic Extract" neutralised 24·15 cc., 24·05 cc. and 24·15 cc. $N/10$ acid. The figure obtained by the older method several months earlier, for the same quantity of extract was 0·7 cc. higher. It was possible, however, for small losses to occur during the interval through exposing the extract to the air whilst removing aliquot portions. A further experiment showed that the whole of the volatile bases can be estimated in a single distillation. In one case 50 cc. alcohol were added to the residual liquid and the resulting solution steam distilled, as before, into a fresh quantity of $N/10$ acid. Only 0·1 cc. further acid was neutralised, making a total of 24·15 cc. for the two distillations.

The residual aqueous liquids consistently required 2·05 cc. $N/10$ acid for neutralisation to phenolphthalein. The figure obtained by the formol titration for the amino-acids several months earlier was 2·1 cc.

The effect of the process upon the composition of added amino-acids was tested. The complex amino-acid solution used in Exp. No. 15 of Table X was used for this purpose. A quantity possessing a total titration value of 3·0 cc. was added to 50 cc. "Alcoholic Extract," and steam distilled according to the method. The titration of the residual liquid gave a result 0·65 cc. too low, showing that a certain amount of change occurred during the short exposure of the added amino-acids to a temperature of 100°. It should be noted that the concentration of free alkali in the residual liquid is greater when more amino-acids are present. In this experiment the volatile bases result was the same as obtained previously, viz. 24·05 cc. It appears therefore that whatever change in the composition of the amino-acids occurred, volatile bases were not produced. Consequently the rapid method, which can be

completed in seven or eight minutes, seems to be very reliable so far as the volatile bases are concerned. Further attempts are being made to obtain an accurate separate figure for the amino-acids.

Summary and Conclusions.

1. Ammonia, primary, secondary and tertiary amines, and basic methylene derivatives of secondary amines, do not form ionisable compounds with phenolphthalein in alcoholic solutions containing water, if the concentration of alcohol is sufficiently high.

2. In aqueous alcoholic solutions of the salts of these bases the acid radicles can be titrated accurately with $N/10$ alkali, using phenolphthalein as indicator, if more than about 80 % alcohol is present.

3. When aqueous-alcoholic solutions of certain amino-acids containing about 85 % alcohol are titrated with standard alcoholic potash, the amino- or imino-groups liberated from their "internal salt" combinations, resemble ammonia and the amines in showing no basicity to phenolphthalein, and the carboxyl-groups are accurately estimated.

4. Other amino-acids, more particularly dibasic amino-acids and proline, give low results when titrated in alcohol under these conditions, possibly owing to loose combination of alcohol with a carboxyl-group, or loose condensation. The subsequent addition of formaldehyde or acetone, however, results in a disturbance of the equilibrium, so that the carboxyl-groups titrate quantitatively.

5. The effect of acetone upon the basicity of the basic groups of amino-acids to phenolphthalein is similar to that of alcohol. The amino-acids tested up to the present have all given practically quantitative results when titrated with $N/10$ alkali in aqueous acetone containing 80–85 % acetone.

6. The guanidine nucleus of arginine behaves differently from other nitrogenous bases or basic groups by titrating quantitatively as a univalent base in alcohol as well as in water. An explanation of this unique behaviour will be given in a further communication. Arginine is neutral to phenolphthalein in alcoholic solutions if sufficient alcohol is present, the carboxyl-group and the guanidine nucleus exactly neutralising one another.

7. A simple general method for accurately estimating the carboxyl-groups of amino-acids, based upon the foregoing conclusions, is described.

8. It has been shown further that the method is capable of much wider application. The total acids (including the carboxyl-groups of amino-acids) whether in the free state or combined with organic bases, can be accurately estimated in alcoholic preparations made from aqueous fluids such as those which contain the products of bacterial growth.

As the titration value is unaffected by the free bases their removal is

unnecessary. Consequently the solutions need no heating, and there is no risk of decomposition.

9. A rapid method for estimating volatile bases is also described.

Finally I wish to thank Mr C. E. Bryant for assistance in carrying out the experiments and for acting as an independent observer of the endpoints of the titrations.

REFERENCES.

Birckner (1919). *J. Biol. Chem.*, **38** [No. 2, June], 245.
Foreman and Graham-Smith (1917). *J. Hygiene*, **16**, 144.
Jodidi (1918). *J. Amer. Chem. Soc.*, **40**, 1031.
Mann (1906). *Chemistry of the Proteids*, p. 211.
Plimmer (1915). *Practical Organic and Bio-Chemistry*, p. 285.
Sörensen (1907). *Biochem. Zeitsch.*, **7**, 79.
Sutton (1900). *Volumetric Analysis*, 8th edition, p. 38.
Walker (1904). *Zeitsch. physikal. Chem.*, **49**, 82.