THE FORMOL TITRATION OF BACTERIOLOGICAL MEDIA

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Received for publication July 2, 1922

The principles involved in the "formol titration" of bacterial cultures or media are the same as those embodied in the methods of Malfatti (1908) and of Henriques and Sörensen (1909) for the titration of urine. They have been applied to the study of bacterial cultures by a number of authors, notably Kendall, Day and Walker (1913), Itano (1916), Berman and Rettger (1918), Foster and Randall (1921), Ayers, Rupp and Mudge (1921), and Kendall (1922). An excess of formalin (approximately 40 per cent formaldehyde) is added to the sample to be titrated. The sample becomes more acid as a result of the following type reactions:

$$\begin{array}{l} R \cdot CH \cdot NH_2 \\ \quad | \\ \quad COOH \end{array} + CH_2O = \begin{array}{l} R \cdot CH \cdot N : CH_2 \\ \quad | \\ \quad COOH \end{array} + H_2O$$

$$4NH_4Cl + 6CH_2O = N_4(CH_2)_6 + 4HCl + 6H_2O$$

The increase in acidity is then titrated with a standard alkali solution. Ammonia, primary amines, and the amino groups of amino-acids, and polypeptids react with formaldehyde. The titration, therefore, represents the sum of these substances.

Sörensen (1907) (1908) has pointed out that the reaction of amino-acids with formaldehyde is a reversible reaction, that an excess of formaldehyde tends to throw the reaction, as expressed in the above equation, from left to right, whereas increasing amounts of water throw the reaction from right to left. Since the methylene derivative of the amino-acid is a stronger acid

than the amino-acid and is therefore more easily titrated, it is obviously desirable to force the reaction from left to right. To do this Sörensen found it necessary to add as much as 10 cc. of formalin to 20 cc. of sample (amino-acid solution), and to titrate with N/5 barium hydroxide or sodium hydroxide. Smaller amounts of formalin were insufficient; larger amounts unnecessary. Workers in bacteriology appear not to have appreciated the importance of this factor. Working with solutions of pure amino-acids Sörensen found it necessary to carry the titration to an end point on the alkaline side of pH 8.2 in order to approximate the theoretical value of some of the amino-acids. He therefore titrated to a deep red color with phenolphthalein as an indicator which he states to have been at about pH 9.0, or to a strong blue color with thymolphthalein which he states to have been at about pH 9.7. Sörensen recognized the presence of carbonates and phosphates as a serious source of error in the formol titrations of amino-acid mixtures containing these substances and to minimize this difficulty titrated with barium hydroxide rather than sodium hydroxide, also adding barium chloride to depress the ionization of the barium carbonate and phosphate formed. The methylene derivatives of some polypeptids and certain of the amino-acids, however, notably tyrosine and phenylalanine, formed insoluble precipitates with barium and for their titration better results were obtained with sodium hydroxide. In the methods of Henriques (1909) and of Henriques and Sörensen (1909) the barium carbonate and phosphate were filtered out, the filtrate neutralized, formalin added, and the acid titrated against sodium or potassium hydroxide.

In developing his method Sörensen made formol titrations of pure solutions of many of the amino-acids and of some peptic, tryptic, and ereptic digests of Witte peptone, casein, and egg albumin. Henriques and Sörensen titrated urines. For the titration of bacterial cultures Itano (1916) followed quite closely the method of Sörensen. Kendall and his associates departed widely from the method of Sörensen in that they used a much larger proportion of water (50 cc.) and a smaller proportion of formalin (5 cc.). Foster and Randall (1921) followed the technic

of Kendall, Day and Walker. Berman and Rettger (1918) departed even more widely from the technic of Sörensen in that they used a total volume of 55 cc. of water and only 2 cc. of formalin. Ayers, Rupp and Mudge (1921) presumably followed Sörensen since they do not mention any modification of the technic. Kendall, Day and Walker and Berman and Rettger departed from Sörensen's method in another respect. They "neutralized" their samples of media to the end point of phenolphthalein, a point which Kendall defines as pH 8.3, before adding the formalin. Sörensen (1908) did not do this. He says, after describing the preparation of the control solution to the "deutliche rote Farbe (zweites Stadium)" of phenolphthalein: "Die zur Untersuchung vorliegenden Lösungen werden bis zu dieser letzten Farbenstärke titriert, indem 20 ccm der Analyse 10 ccm Formolmischung zugesetzt werden, und gleich darauf n/5 Barytlauge bis Rotfärbung, ..."

In the French edition (1907) of his article the meaning is equally clear. He says: "Ensuite on titre jusqu'à la même intensité de coloration les liqueurs à examiner; à cet effet, on ajoute à 20 cc. de la liqueur 10 cc. du mélange de formol et immédiatement après, en agitant bien, la solution de baryte au 1/5, jusqu'à ce que le liquide devienne rouge, ..."

It seems worth while quoting the above passages verbatim because the description of Sörensen's method in certain text books of physiological chemistry would lead one to believe that the solution to be analyzed should be titrated to the color of phenolphthalein produced in the second stage of the control before the formalin is added.

For the titration of solutions of pure amino-acids Sörensen (1907) (1908) apparently carried out no preliminary neutralization or adjustment of hydrogen ion concentration, though when it was necessary to add alkali to get the substance into solution (e.g. tyrosine and aspartic acid) the amount of alkali added was considered in the computation of the formol titration. Later Henriques and Sörensen (1909) (1910) pointed out that the neutral point of the amino-acids is at about pH 6.8 and that this therefore should be the starting point for the titration. They

brought their samples to this reaction by means of a specially prepared litmus paper. Northrop (1921) adopted pH 7.0 with neutral red as an indicator for the starting point of the titration of hydrolyzed gelatin. H. Jessen-Hansen in Abderhalden's Handbuch der biochemischen Arbeitsmethoden, vi, 262, gives a very good discussion of the importance of this factor. Theoretically the isoelectric point should be the point of departure for the titration of amino-acids. Fortunately for our purpose the isoelectric points of the various amino-acids are so close together that their isoelectric zones (Michaelis, 1914, p. 40) overlap and pH 6.8 or 7.0 may be chosen as the ideal starting point for the titration of mixtures of amino-acids and ammonium salts.

The experiments and curves which follow serve to illustrate the importance of choosing the correct hydrogen ion concentrations for the initial and end points of the formol titrations. In bacteriological culture fluids we encounter not only mixtures of amino-acids and ammonium salts but also peptones, polypeptids, fatty acids, carbonates, phosphates, and carbohydrates. The reaction may be acid or alkaline. There are also the disturbing factors of color and turbidity. By known methods of precipitation, filtration, etc. it is possible to eliminate the disturbing factors in such a mixture and to make an accurate formol titration. The present paper is an attempt to analyze the factors involved and to reduce the formol titration of bacteriological media and cultures to its simplest terms.

Phenol red or thymol blue have been used as indicators. The end point is judged by comparison with colorimetric hydrogen ion standards in a comparator block. This not only leads to much greater accuracy in judging the end point but makes it possible to use a color screen of the material being titrated, doing away with the necessity for using artificial colors in a control as was done by Sörensen. Since this work has been in progress Northrop (1921) has also made similar use of the comparator block and colorimetric standards. The titration is carried on in large test tubes containing 1 cc. of sample and 9 cc. of distilled water. The alkali used is N/20 NaOH. For none of the media or cultures titrated to date has it been necessary to use more than 3 cc. of the alkali solution.

As a result of numerous experimental titrations conducted at various hydrogen ion concentrations it is found that optimum results are obtained when 8 cc. of formalin are added to the 10 cc. of diluted sample. Lesser amounts of formalin give lower titrations. It is desirable to use no more formalin than necessary because of the high buffer effect of the formalin itself which results in a poor end point if the titration is carried beyond pH 8.0. More than 8 cc. of formalin have been found unnecessary. The effect of using different amounts of formalin is illustrated in table 1.

In the titrations hereafter reported it is to be understood that 8 cc. of formalin were added to 10 cc. of the diluted sample.

TABLE 1

Effect of various amounts of formalin on the formol titration

SAMPLE (1 cc. + 9 cc. H_2O)	AMOUNT OF FORMALIN			
	1 cc.	5 cc.	8 cc.	10 cc.
Alanine	1.16*	1.94	2.0	2.0
Bouillon	0.9	1.03	1.09	1.09

* The figures represent cc. of N/20 NaOH required for the titration of 1 cc. of sample, the formalin blank[2] having been determined for each amount of formalin and subtracted.

There remain to be determined the limits of hydrogen ion concentration between which titration shall be conducted. If one were to titrate an amino-acid directly against sodium hydroxide, i.e , without the use of formalin, he should start the titration at the isoelectric point of the amino-acid and end it at the point of complete neutralization of the amino-acid. In the case of glycine this would be from pH 6.1 to about pH 11.3. However in the case of glycine and other amino-acids the isoelectric point is in a broad isoelectric zone extending in the case of glycine from about pH 5.0 to pH 7.5 or 8.0. Within this zone the degree of dissociation (a) is very slight and even at pH 8.0 is less than 2 per cent.

$$a = \frac{k_a}{k_a + (H^+)} = \frac{1.8 \times 10^{-10}}{1.8 \times 10^{-10} + 1 \times 10^{-8}} = \frac{1.8}{101.8} \text{ at pH 8.0}$$

If a pure monocarboxylic amino-acid is dissolved in distilled water the solution will be found to have a hydrogen ion concentration at or near the isoelectric point, a fact also noted by Eckweiler, Noyes and Falk (1921). If it is titrated with alkali and the titration curve plotted with amounts of alkali added as abscissae and hydrogen ion exponents as ordinates, as was done in the curves to the left in plate 1, the curve will be seen to drop almost vertically towards the alkaline side as the first drops of alkali are added. This represents a portion of the isoelectric zone. Asparagine (plate 2) behaves as a monocarboxylic amino-acid since in this amide one of the carboxylic groups of aspartic acid is in combination with an amide group. Since by the formol titration it is the carboxyl radical and not the amino radical which is titrated it is important that the monoamino-dicarboxylic acids be converted into mono-carboxylic acids, otherwise their titer will be doubled. This may be accomplished by titrating them with sodium hydroxide to the isoelectric point (or to within the isoelectric zone) of their mono-sodium salts. This zone is represented by the nearly vertical portion of the curves in plate 2. To titrate the nitrogen equivalent of the mono-amino-dicarboxylic acids it is therefore necessary to start the titration within the isoelectric zone of their mono-sodium salts and this zone lies within the same region as that of the mono-carboxylic amino-acids.

Within the isoelectric zone a very minute amount of acid or alkali produces a marked change in hydrogen ion concentration. It makes scarcely any difference in titration at what point we start provided it is within the isoelectric zone. The sodium salts of the amino-acids are not titratable after the addition of formalin since they react with formaldehyde to produce not acids but sodium salts of the methylene derivatives of amino-acids. In a solution of glycine at pH 8.4 ($a = \frac{1.8}{41.8}$) about 4.3 per cent of the glycine is present as the sodium salt. If formalin is added at this point the reaction becomes more acid, or rather less basic, because of the formation of methylene derivatives. However there has already been added sufficient alkali to neutralize 4.3

per cent of the methylenglycine and unless this is taken into account, as was done by Sörensen when he dissolved amino-acids in alkali, the formol titration will fall short by a corresponding amount. Before the formalin is added, mixtures of amino-acids, protein digests, bacterial cultures, etc., should be brought to a reaction within the isoelectric zones of all the amino-acids present if this is possible or unless other considerations make it necessary to compromise in regard to this point. We will call this the initial point of the titration. The end point of the titration should be within what may be called the "zone of neutralization" of the amino-acid if it is titrated directly or within the zone of neutralization of the methylene derivative of the amino-acid if formalin has been added. The term "zone of neutralization" will be used to indicate that portion of the titration curve of an acid and base which extends, on either side of the neutral point, nearly vertically to the abscissa with increasing or decreasing hydrogen ion concentrations plotted as ordinates. The neutral point is the point of inflection of the curve within the zone of neutralization and is reached when just sufficient alkali has been added to convert all of the acid into its alkali salt. The neutral point in the titration of the methylene derivative of an amino-acid is at a higher hydrogen ion concentration than that in the titration of the amino-acid itself and it is upon this fact that the usefulness of the formol titration depends. If formalin is added to solutions of various amino-acids and these mixtures are then titrated with sodium hydroxide, the curves plotted on the right in plates 1 and 2 are obtained. In these curves an almost vertical portion is harder to recognize. This is because of the great excess of formalin in the mixtures titrated. Of the 8 cc. of formalin added to each sample a very small fraction of 1 cc. actually reacts with the amino-acid to form the methylene derivative. The remainder exerts a powerful buffer effect on the alkaline side of pH 8.0. Commercial formalin is acid in reaction. It has been our practice to add to it in preparation for each day's work sufficient normal NaOH to reduce the acidity to about pH 5.0, then to titrate 8 cc. of it plus 10 cc. of distilled water to pH 9.0 or beyond with N/20 NaOH, to plot the titration curve, and

use it for the day's experiments. In plates 1, 2, and 3 points determined on these curves are indicated by crosses. If titration values on the formalin curve are subtracted from values at the same hydrogen ion concentration on the amino-acid plus formalin curve the differences may be plotted as a resultant curve lying between the other two. This resultant curve should be the titration curve of the methylene derivative of the amino-acid. Each one of these resultant curves does reach a nearly vertical position which is the zone of neutralization of the methylene derivative and is the end point of the titration. For each of the substances titrated this zone may be said to begin on the acid side at about the hydrogen ion concentration here listed.

	pH
Glycine	6.8
Phenylalanine	7.6 or 8.0
Asparagine	6.0
Glutamic acid	8.0
Alanine	8.0
Tyrosine	7.6 or 8.0
Aspartic acid	8.0

A similar zone is found in the resultant curve of the formol titration of ammonium salts (plate 3). In these cases the resultant curve represents the titration of hydrochloric, lactic phosphoric, or carbonic acid. The zones may be said to begin at the following hydrogen ion concentrations.

	pH
Ammonium chloride	6.0
Ammonium phosphate	8.0
Ammonium lactate	6.0
Ammonium carbonate	7.0

It will be noted that before the resultant curves reach pH 9.0 their course becomes somewhat irregular or uncertain. This is probably because in the titration of formalin and of formalin plus amino-acids the buffer effect of both substances is so great after they pass beyond pH 8.5 that the end point judged colorimetrically is a very broad and indefinite one. The effect of this is indicated in the cases of glycine, alanine, and asparagine (plates 1 and 2) by shaded regions. The end points of the titrations

might have been taken to lie at any point within these regions. The experimental or technical error is therefore quite large if the titration is carried much beyond pH 8.0 and is in our opinion too large for formol titration by the colorimetric method if carried beyond pH 9.0. The indicators used by us in these regions were phenol red and thymol blue. We found the end point with phenol red at pH 8.0 a very sharp one.[1] At pH 8.0 all of the substances titrated after the addition of formalin had reached the zone of neutralization. This then might be taken as the maximum hydrogen ion concentration at which the formol titration might be completed. An end point of pH 8.2 or 8.4 might be a little better were it not for certain other factors peculiar to the titration of bacteriological media which are discussed below. On the other hand the curves show that pH 8.0 is a little too alkaline to be within the isoelectric zones of some of the amino-acids and ammonium salts. The ideal starting point for formol titration of mixtures of amino-acids would therefore be at a higher hydrogen ion concentration. A hydrogen ion concentration of pH 6.8 as recommended by Henriques and Sörensen (1909) (1910) or of pH 7.0 as employed by Northrop (1921) would serve very well.

However our problem is a special one in that bacteriological media and especially bacterial cultures in such media are more than mere mixtures of amino-acids. They contain among other things volatile and non-volatile fatty acids, phosphates and carbonates, and these substances, especially fatty acids and carbonates together with amino-acids and ammonium salts may change in amount during the growth of the culture. What influence do these substances exert within the range of hydrogen ion concentration chosen for the formol titration? At pH 7.0 the volatile fatty acids are practically neutralized so that they do not enter into a titration with sodium hydrate from pH 7.0 to pH 8.0. The phosphates and carbonates exert large buffer effects between pH 7.0 and pH 8.0, and the amount of alkali

[1] Through the kindness of Dr. van Slyke and Dr. Hastings of the Hospital of the Rockefeller Institute for Medical Research in New York City our color standard at this hydrogen ion concentration was checked electrometrically and found to be correct.

with which they are capable of combining will appear as an error in the formol titration between these limits. If therefore the solution to be titrated is brought to a certain hydrogen ion concentration (e.g., pH 7.0) formalin added and the mixture titrated to a lower hydrogen ion concentration (e.g., pH 8.0 or 8.4) it is absolutely necessary to get rid of the phosphates and carbonates. This may be done by precipitation with barium and filtration.

The following method has given the most accurate results with bacteriological media and with mixtures of amino-acids, phosphates and carbonates.

Method A. Measure out accurately with an Ostwald pipette 2 cc. of culture medium. Add from a burette exactly 2 cc. of N/5 or stronger NaOH. Add a small piece (about 0.2 gram) of barium chloride. Shake to dissolve the barium chloride and allow to stand for a few minutes. Pour onto a small dry paper filter.

With the Ostwald pipette measure out 2 cc. of the filtrate into a large test tube (1 inch in diameter) such as can be used in the comparator block. Caution: Do not blow through the pipette used for measuring the sample since the CO_2 from the breath will cause the fluid to become cloudy.

Add 5 drops of phenol red solution and sufficient N/5 HCl to bring the reaction of the sample near pH 7.0 and then sufficient distilled water to bring the contents of the tube to about 10 cc. which may be judged by a mark on the side of the tube. Bring the reaction of the sample to pH 7.0 with N/20 HCl or NaOH, using the comparator block with 1 cc. of medium plus 9 cc. of water as a color screen behind the colorimetric standard.

Add 4 drops more of the phenol red solution and 8 cc. of formalin.[2] Titrate with N/20 NaOH to pH 8.0 or 8.4. From this result subtract

[2] It is our experience that if formalin is made alkaline it does not "keep" well, *i.e.*, the hydrogen ion concentration increases slowly but appreciably within two hours. If, however, it is made slightly acid it does not change appreciably during the working day. Instead of adjusting the reaction of the formalin to pH 8.0 or 8.4 before adding it to the sample of medium we prefer to prepare it as follows: To a sufficient quantity for the days' work is added normal NaOH (usually less than 1 cc. per 100 cc. of formalin) until the reaction is between pH 5.0 and pH 7.0. The formalin blank consists of 8 cc. of this formalin plus 10 cc. of distilled water titrated against N/20 NaOH to pH 8.0 or 8.4 (the end point chosen for the formol titrations).

the titration of the formalin blank.[2] The remainder multiplied by 5 is the formol titration expressed in terms of per cent normal, *i.e.*, the percentage normality of substances reacting with formaldehyde to produce titratable acids.

In the formol titration of bouillon we have obtained exactly the same values when titrating to pH 8.0 as at pH 8.4 whereas the colorimetric end point at pH 8.0 is somewhat sharper than at pH 8.4 because of the greater buffer effect of the formalin at pH 8.4.

A briefer method than the above, one which requires less material and which gives almost identical results even in the presence of large amounts of carbonates and phosphates in bouillon, is as follows:

Method B. With an Ostwald pipette measure out 1 cc. of the medium or culture into each of two large test-tubes (1 inch in diameter) such as can be used in the comparator block. To each tube add 9 cc. of ammonia-free distilled water or better sufficient water to bring the contents of both tubes to the same level. One of the tubes serves as a color screen in the comparator block. To the other tube, hereafter referred to as the sample, add 5 drops of phenol red (the same proportion of indicator as is contained in the colorimetric hydrogen ion standards).

Bring the reaction of the sample to pH 8.0 by the cautious addition of of N/20 NaOH or HCl[3] as needed. A few drops are usually sufficient.

To the sample add 4 drops more of phenol red and then to both the sample and the color screen tubes add 8 cc. of formalin.[2] Twirl the sample tube just sufficiently to mix the formalin with the sample and as quickly as possible but with as little agitation as possible, add from a burette[3] N/20 NaOH until the pink color of the indicator is visible, then more deliberately titrate to pH 8.0. After subtracting the titration of the formalin blank the result multiplied by 5 is the formol titration expressed in terms of per cent normal. By multiplying the latter result by 14 the result may be expressed as milligrams of nitrogen per 100 cc. of medium.

[3] The burette used should be finely graduated so that it can be read in hundredths of a cubic centimeter. The N/20 NaOH and HCl used should contain phenol red in the same concentration as is present in the sample and in the colorimetric standards, a suggestion adopted from Hurowitz, Meyer, and Ostenberg (1915).

The method above described resembles that of Kendall, Day and Walker (1913) and that of Berman and Rettger (1918) in that the sample is brought to a certain hydrogen ion concentration before formalin is added and titrated back to the same hydrogen ion concentration after the addition of the formalin, but it differs from them in other respects. Kendall, Day and Walker allowed the sample of medium to stand thirty minutes after the addition of formalin before proceeding with the titration. We have found this not only unnecessary but distinctly bad because of the loss of CO_2 into the atmosphere. The CO_2 may be removed before the formalin is added in which case any ammonium carbonate which was present is transformed into other ammonium salts and there will be no further loss of CO_2 after the formalin is added, but the escape of CO_2 after the formalin is added means a decreasing titratable acidity and hence lowers the formol titration. The CO_2 must either be removed before adding the formalin or must be kept in solution until the titration is finished. If in the presence of carbonates it is desired to allow the mixture of formalin and sample to stand for a few minutes it should first be brought to an alkaline reaction. The effect of the escape of CO_2 into the air is illustrated by the formol titration curve of ammonium carbonate in plate 3. This titration was carried out with four different indicators in four different samples of ammonium carbonate solution. Formalin was added to the first sample (methyl red) and it was titrated deliberately from pH 5.2 to 5.6. Formalin was added to a second sample (brom cresol purple) and it was titrated from pH 6.0 to 6.8. Similarly a third sample (phenol red) was titrated from pH 7.0 to 8.4. The fourth sample (thymol blue) was titrated from pH 8.8 to 9.0. It will be noted that as the titration of each sample progressed the curves ending on the acid side of pH 7.0 fell short, so as to produce breaks or steps in the entire curve at A and B, a condition which did not occur in the absence of carbonates, as is illustrated by the curves of the other substances similarly titrated. We attribute these breaks to loss of CO_2. This is further illustrated in table 2.

TABLE 2

Illustrating the effect of loss of CO_2 during the formal titration

SAMPLE	PROCEDURE	RESULT
Glycine	Titrated deliberately	2.53*
Glycine + 1 per cent $NaHCO_3$	Titrated several minutes after addition of formalin	2.34
Glycine + 1 per cent $NaHCO_3$	Titrated quickly after addition of formalin	2.52
Glycine + 1 per cent $NaHCO_3$	Titrated under oil	2.55
Glycine + 1 per cent NaH_2PO_4	Titrated deliberately	2.54

* In this and the succeeding tables the results are expressed as cubic centimeters of N/20 NaOH required for the titration of 1 cc. of sample, the formalin blank[2] having been subtracted.

TABLE 3

Formol titrations in the presence of carbonates and phosphates

SAMPLE	INITIAL POINT OF TITRATION	END POINT OF TITRATION	RESULT
	pH	*pH*	
Bouillon only (containing only the phosphates and carbonates which are native to meat infusion bouillon)	7.0	8.0	1.35
	7.0	8.4	1.35
	8.0	8.0	1.07
	8.4	8.4	0.96
Bouillon + 1 per cent $NaHCO_3$ and 1 per cent Na_2HPO_4 (titrated quickly)	7.0	8.0	1.9
	8.0	8.0	1.06
Bouillon + 1 per cent $NaHCO_3$ and 1 per cent Na_2HPO_4 (carbonates and phosphates precipitated by $BaCl_2$ and filtered out)	7.0	8.0	1.13
Glycine only	7.0	8.0	1.24
	8.0	8.0	1.22
Glycine + 1 per cent $NaHCO_3$ and 1 per cent Na_2HPO_4 (titrated quickly)	8.0	8.0	1.22
Glycine + 1 per cent $NaHCO_3$ and 1 per cent Na_2HPO_4 (carbonates and phosphates precipitated by $BaCl_2$ and filtered out)	7.0	8.0	1.26

By bringing the reaction back to alkaline as quickly as possible after the addition of formalin the loss of CO_2 into the air is minimized. By adjusting the reaction of the sample to pH 8.0 and carrying the final titration to the same point the buffer effect of phosphates, carbonates, or other buffer substances in the medium is eliminated. That by these means the necessity for removing the phosphates and carbonates by precipitation is obviated is illustrated in tables 2 and 3. Other means have been tried, such as preliminary acidification and boiling or aeration to get rid of the CO_2, or titration under a layer of oil, but the technic described above is almost if not quite as efficient and much simpler.

From a study of the titration curves of pure amino-acids and ammonium salts already described it was concluded that optimum results were obtained by adjusting the reaction of the sample to pH 6.8 or 7.0 then adding the formalin and titrating to pH 8.0 or 8.4. However, with bouillon, and particularly if phosphates or carbonates have been added, titration between these limits of hydrogen ion concentration is impossible as is also illustrated in table 3. To eliminate the buffer effect of these substances the formol titration must start and end at the same hydrogen ion concentration. It is therefore imperative to determine what this hydrogen ion concentration should be. For each of the pure substances titrated formol titrations were made between such limits as pH 5.6 to 5.6, pH 6.8 to 6.8, pH 7.0 to 7.0, pH 7.6 to 7.6, pH 8.0 to 8.0, pH 8.4 to 8.4, and pH 9.0 to 9.0. These titrations are indicated by small triangles in plates 1, 2 and 3.[4] The results are in strict agreement with what might be deduced from the character of the other curves plotted. If the isoelectric zone of the amino-acid overlaps a portion of the zone of neutralization of the methylene derivative (resultant curve) (i.e., if the two curves fall nearly vertically at

[4] For all of the solutions, the titrations of which are plotted in plates 1, 2 and 3, total nitrogen or ammonia determinations were made. On the assumption that these determinations represent 100 per cent of the substance in solution the curves are so adjusted that a perfect formol titration (*i.e.*, titration of 100 per cent of the substance) should require 2 cc. of N/20NaOH. A solution of this concentration would be one-tenth normal.

the same hydrogen ion concentration) the formol titration gives practically perfect results when carried out between limits of hydrogen ion concentration within the overlapping portion of these zones. For example the isoelectric zone of glycine (plate 1) and the zone of neutralization of its methylene derivative (resultant curve) overlap from about pH 6.8 to 8.0 and practically 100 per cent of the glycine may be determined by formol titration from pH 6.8 to 6.8 or from pH 8.0 to 8.0. However on either side of these extremes, as shown by the triangles at pH 5.6, 8.4 and 9.4, lower values are obtained. In the case of alanine there is only slight overlapping of the zones at pH 8.0 to perhaps 8.4. In the case of asparagine, ammonium chloride and ammonium lactate there is overlapping from about pH 6.0 to 7.0. In the case of the other amino-acids titrated there is no actual overlapping of the zones, consequently with these substances all formol titrations which begin and end at the same hydrogen ion concentration are too low, and if we are to use the method under consideration the hydrogen ion concentration at which a maximum titration is obtained must be found. Maximum titration values for certain amino-acids were obtained at the following hydrogen ion concentrations; phenylalanine, pH 7.6; tyrosine, pH 8.0; aspartic acid, pH 8.4; glutamic acid, pH 8.0 to 8.4. We did not have the difficulty experienced by Sörensen (1907) (1908) of obtaining results that were too high in the formol titration of tyrosine. With ammonium phosphate and ammonium carbonate another phenomenon is encountered; the line of the formol titrations starting and ending at the same hydrogen ion concentration crosses the titration curves of phosphoric and carbonic acids (resultant curves). The formol titration of ammonium phosphate at points to the acid side of pH 7.8 leads to results which are too high while titration at points to the alkaline side of pH 8.0 gives low results. Perfect results are obtained at pH 7.8 or 8.0. Formol titration of ammonium carbonate did not yield 100 per cent of the ammonia value probably because of the escape of some of the CO_2 during titration, but maximum results (at least 90 per cent of the theoretical) were obtained at pH 6.0, 7.0, or 8.0.

If it is desired to perform a formol titration of a mixture of amino-acids and ammonium salts from a selected hydrogen ion concentration back to the same hydrogen ion concentration, as here proposed, the optimum hydrogen ion concentration will evidently depend upon the relative proportions of the various amino-acids and ammonium salts present. This optimum hydrogen ion concentration has been determined empirically for solutions of "aminoids," peptone and standard meat infusion bouillon (see table 4).

TABLE 4

Formol titrations of bacteriological media at various hydrogen ion concentrations

MEDIUM	THE INITIAL AND END POINTS OF TITRATION				
	pH 7.2–7.2	pH 7.6–7.6	pH 8.0–8.0	pH 8.4–8.4	pH 9.0–9.0
1 per cent beef aminoids*.....	0.86	0.9	0.95	0.79	0.64
1 per cent casein aminoids*.....	0.68	0.71	0.74	0.65	0.36
1 per cent Fairchild peptone.....	0.68	0.67	0.68	0.52	0.39
	(pH 7.0–7.0)				
Veal bouillon 1502.....	1.04	1.12	1.11	1.04	
Veal bouillon 1502 + 1 per cent NaH_2PO_4.....	1.35	1.23	1.10	1.04	
Veal bouillon 1502 + 1 per cent $NaHCO_3$.....	0.81	1.04	1.07	0.99	

* Arlington Chemical Co. product.

The "aminoids" gave maximum formol titrations at pH 8.0. Fairchild peptone gave maximum results between pH 7.2 and pH 8.0. The veal infusion bouillon gave maximum titrations at pH 7.6 and pH 8.0. In the case of other samples of bouillon we have found the maximum more often at pH 8.0 than at pH 7.6. When $NaHCO_3$ or NaH_2PO_4 was added to the bouillon practically the same result was obtained at pH 8.0, but in the phosphate bouillon higher titrations were obtained at pH 7.6 and at pH 7.0. This phenomenon is related to a similar peculiarity of the ammonium phosphate curve (plate 3) already commented upon. This fact and the comparison with the titrations of the plain bouillon and other substances serve to indicate that the formol titration of bouillon from pH 8.0 to 8.0 gives the most

nearly correct value, a value very close to that which may be obtained after removal of the phosphates and carbonates. In the case of pure amino-acids and ammonium salts the formol titration from pH 8.0 to 8.0 yields from 90 to 100 per cent of the theoretical value, depending upon the individual substance titrated.[5]

It will be of interest to know how the results so obtained compare with those obtained by the methods of Kendall, Day and Walker (1913), Foster and Randall (1921), Kendall (1922),

TABLE 5

Comparison of the results of formol titration by the methods of Kendall, Berman and Rettger, and the method here described

SAMPLE	METHODS OF		
	Kendall	Berman and Rettger	The author
Glycine	2.27*		2.58
Asparagine	0.86		1.11
Veal bouillon	0.86	0.65	1.07

* For purpose of comparison all the results are reduced to the same terms. The figures represent cc. of N/20 NaOH required for the titration of each 1 cc. of sample, the formalin blank[2] having been subtracted in the author's method, the formalin having been "neutralized" to phenolphthalein in the methods of Kendall and of Berman and Rettger.

and Berman and Rettger (1918). The results recorded in table 5 serve to show that the results of Kendall's method are probably at least 20 per cent too low and those of Berman and Rettger even lower. Assuming that the ammonia determinations in the work of these authors were correct it will be seen that when the ammonia nitrogen was subtracted from the formol nitrogen the resulting amino nitrogen value probably involved an error even greater than that indicated by the formol titration alone. Three factors must have conspired to produce these

[5] Eckweiler, Noyes and Falk (1921) have published a titration curve of glycylglycine which is said to be similar to that of other simple dipeptides. It is to be noted that according to this curve the formol titration of these substances from pH 8.0 to 8.0 would introduce a larger error than that found in the titration of simple amino-acids. However, in the case of standard meat infusion bouillon at least, the sum of errors is not a large one.

errors: (1) the use of too much water and too little formalin, (2) the titration from pH 8.3 or 8.4 (the end point of phenolphthalein) back to pH 8.3 or 8.4, and (3) the loss of CO_2 from the sample after formalin had been added.

In criticism of the method of Sörensen it was claimed by de Jager (1909) that a mixture of glycine and ammonium salt gave a formol titration which was lower than the sum of the formol titrations of the glycine and ammonium salt made separately. Henriques and Sörensen (1910) found this of little consequence

TABLE 6

Formol titration of mixtures of an amino-acid and an ammonium salt

SAMPLE	FORMOL TITRATION (pH 8.0 TO 8.0)
1 cc. glycine solution	2.53
1 cc. ammonium lactate solution	1.37
Sum	3.90
Mixture of 1 cc. glycine solution and 1 cc. ammonium lactate solution	3.91

TABLE 7

Formol titration of an amino-acid added to bouillon

SAMPLE	FORMOL TITRATION (pH 8.0 TO 8.0)
1 cc. glycine solution	1.17
1 cc. bouillon	1.09
Sum	2.26
Mixture of 1 cc. glycine solution and 1 cc. bouillon	2.26

in the titration of urines. In table 6 it is shown that this is not a disturbing factor in the method here described.

In the growth of bacterial cultures amino-acids are often liberated from protein substances in the medium. It is therefore of direct interest to know whether the amount of such acid liberated may be actually measured by the formol titration. The formol titration of bouillon before and after the addition of a titrated amount of glycine indicates that it can be so measured (see table 7).

SUMMARY

The influence of certain factors involved in the formol titration of amino-acids and ammonium salts is illustrated. Their titration curves are plotted.

Serious errors in current methods of performing the formol titration of bacteriological media are pointed out.

It is shown that the formol titration of bacteriological media presents a special problem. Simple methods of performing this titration are described, methods requiring very small samples of media. Method A is a modification of the technique of Henriques and Sörensen for the formol titration of urine and involves the removal of carbonates and phosphates by precipitation with barium. Method B is almost as accurate as A, yielding results which probably represent 94 per cent of the amino-acids and ammonia present, and does not require the removal of carbonates and phosphates.

It is a pleasure to express my gratitude to Dr. Paul E. Howe of this Department of the Rockefeller Institute for many valuable suggestions and criticisms during the course of the work, and also to Dr. D. D. van Slyke and Dr. J. H. Northrop of the Rockefeller Institute in New York City for criticism of the manuscript.

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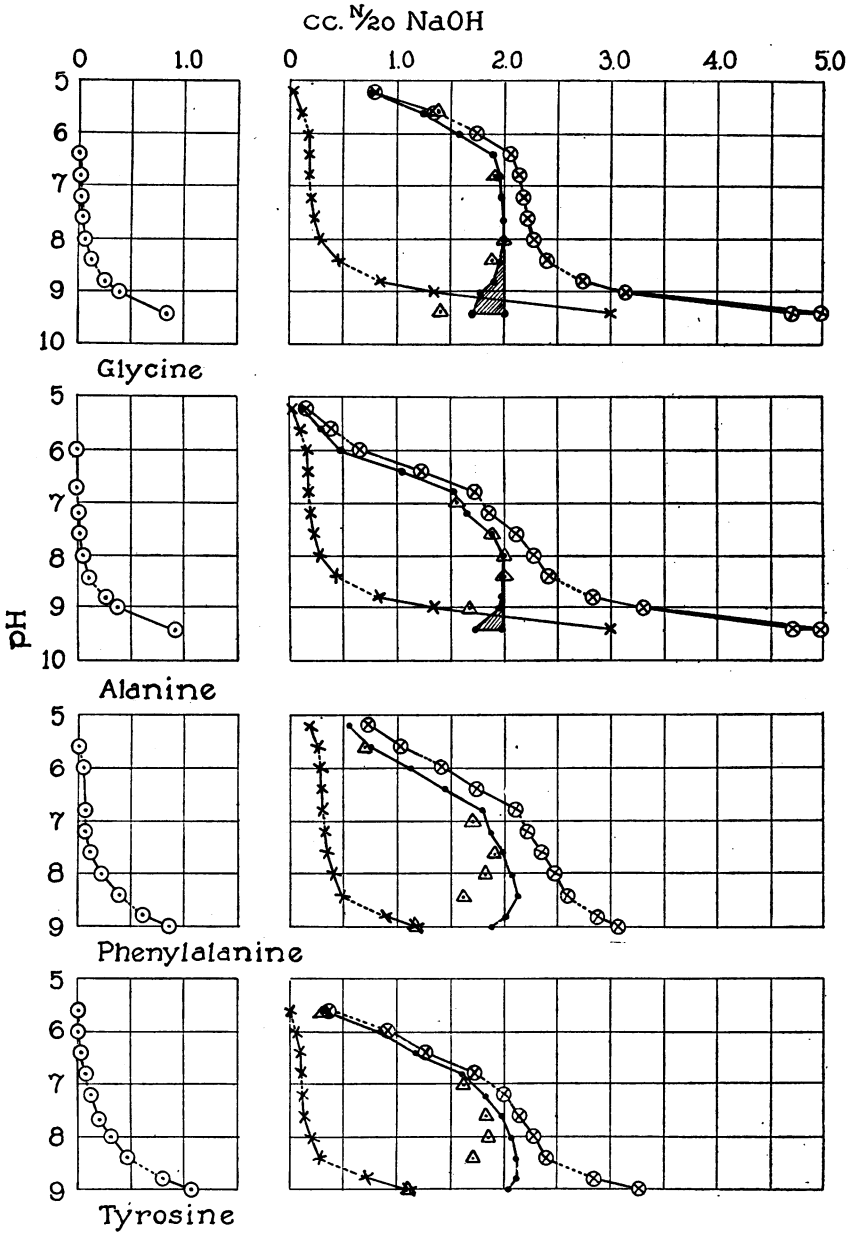
EXPLANATION OF PLATES

1. —⊙—⊙— titration curve of 1 cc. of sample plus 9 cc.water.
2. —×—×— titration curve of 8 cc. formalin plus 10 cc. water.
3. —⊕—⊕— titration curve of 1 cc. sample plus 9 cc. water and 8 cc. formalin.
4. —•—•— resultant curve obtained by subtracting values on curve 2 from those on curve 3.
5. △ formol titrations.

Note: The substance titrated, i.e., the sample, is indicated by the name printed beneath each set of curves.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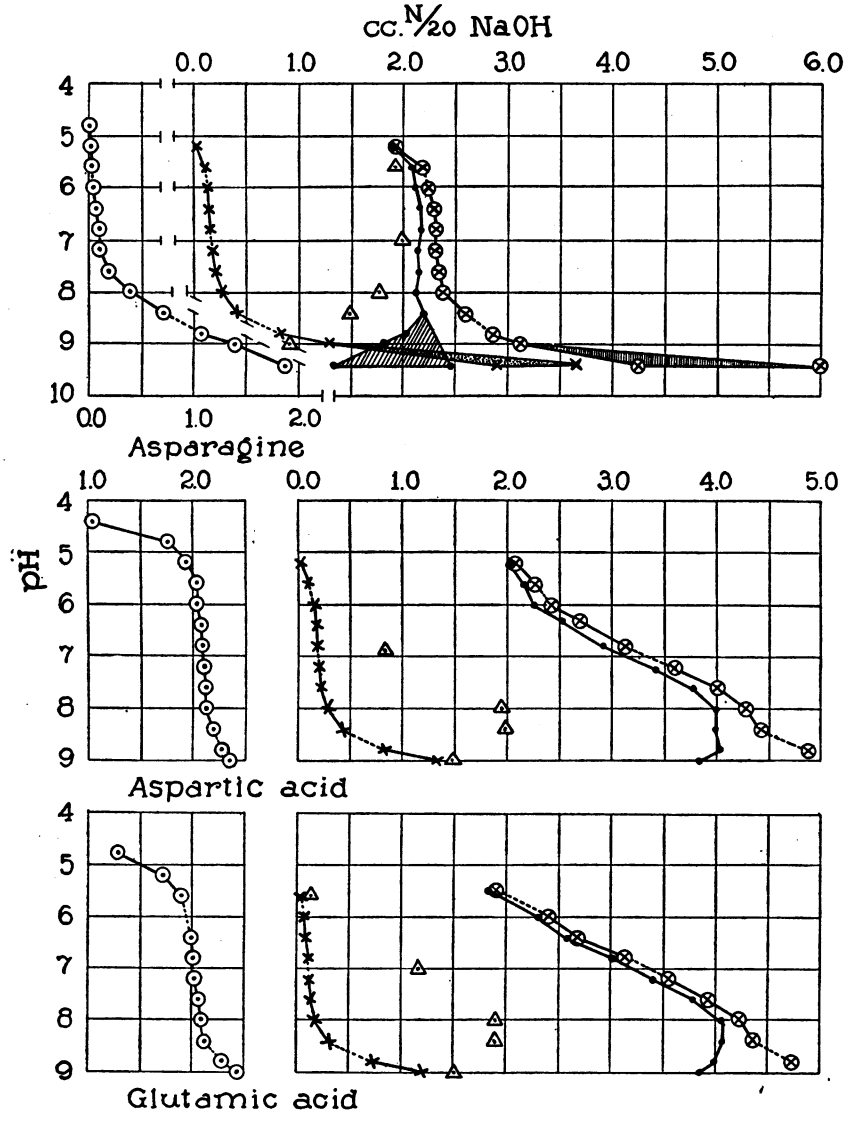
0
1.0
0
1.0
2.0
3.0
4.0
5.0
pH
5
6
7
8
9
10
Glycine
Alanine
Phenylalanine
Tyrosine

PLATE 1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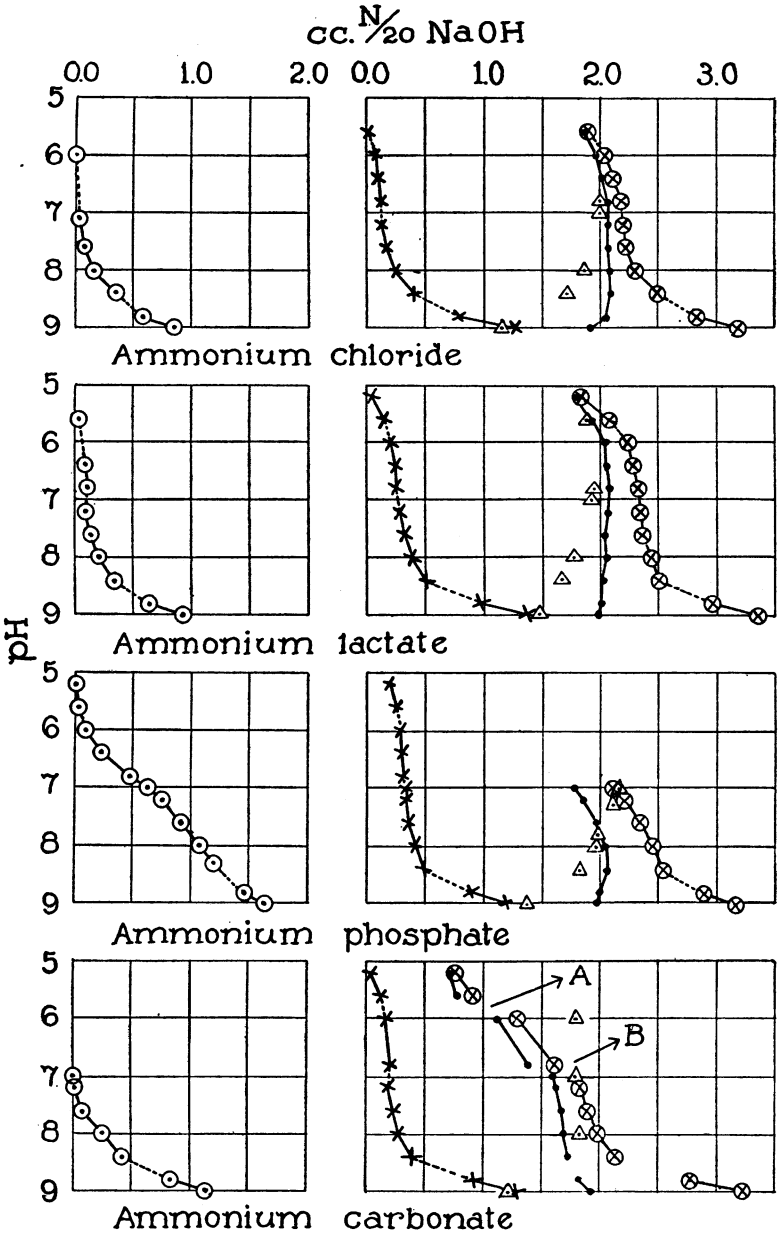
0.0
1.0
2.0
3.0
4.0
5.0
6.0
pH
4
5
6
7
8
9
10
Asparagine
Aspartic acid
Glutamic acid

PLATE 2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0.0
1.0
2.0
0.0
1.0
2.0
3.0
pH
5
6
7
8
9
Ammonium chloride
Ammonium lactate
Ammonium phosphate
Ammonium carbonate
A
B

PLATE 3