The very low value obtained with sea water from the breakwater at Portland Bill remains inexplicable.

It is a pleasure to acknowledge my indebtedness to Mr. A. A. Smales, Head of Analytical Chemistry Group, Chemistry Division, A.E.R.E., who proposed the problem and made several valuable suggestions during the course of the work.

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Formol Titration: An Evaluation of its Various Modifications

By W. H. TAYLOR

The confusion of procedure and interpretation surrounding the formol-titration method of determining amino-acid nitrogen are reviewed, its origins are traced and the principles underlying the application of the method in various experimental circumstances are examined.

Under conditions that simulate protein hydrolysis and with a final formaldehyde concentration of 6 to 9 per cent., evidence from indicator and potentiometric titrations supports the conclusions that (*a*) in determining the degree of proteolytic hydrolysis a direct titration from the pH of hydrolysis to pH 8·5 in formaldehyde gives reasonably quantitative and reproducible results, (*b*) indirect procedures do not yield reproducible results, (*c*) with preliminary neutralisation to pH 9 amino acids are under-estimated slightly and peptides severely, and (*d*) with preliminary neutralisation to pH 7 peptides are under-estimated slightly, but amino acids are not.

Although formol titration has been widely used to determine the extent of proteolytic hydrolysis and to determine amino acids in biological fluids such as urine, published accounts of the method in original papers and in authoritative works of reference vary widely and may cause confusion to those who wish to use it for the first time. The various procedures that are in use are summarised in this paper and the principles underlying their application in various experimental circumstances are examined. A comparison of the accuracy of these procedures, when used for determining the degree of proteolysis, is attempted experimentally for the first time.

Summary of technical procedures of formol titration

There are two chief methods of carrying out formol titration, which may be called direct and indirect. Most works of reference mention only one of these, but both are described by Brown,[1] Jessen-Hansen,[2] Cole,[3] Van Slyke and Kirk[4] and Richardson.[5]

Direct method—

Neutralised formaldehyde is added directly to the unneutralised solution under investigation, which is then titrated with alkali to its final end-point.[3,6,7,8,9,10,11,12] The procedure

may be illustrated by reference to the titration curves of single amino acids (see Figs. 1, 2 and 3, p. 490). Titration starts at the pH of solution in formaldehyde and follows curve A to the chosen end-point, which is usually near pH 9, with phenolphthalein as indicator. Cole[3] and Bodansky and Fay[11] prefer lower end-points of pH 8·3 and 8·5, respectively, but with use of the same indicator. The formaldehyde solution is neutralised to the pH of whichever end-point is selected. After subtraction of an appropriate blank, the direct titration is taken as a measure of the amino acids and peptides present. The composition of the blank or control solution varies. In determining the degree of proteolysis, when only the increase in amino groups is required, Bodansky and Fay[11] and Davis and Smith[12] used or recommend a sample of the digestion mixture at zero time. Sorensen and Jessen-Hansen[7] and Plimmer,[9] however, describe a technique for applying formol titration to coloured solutions, in which a blank of an equivalent volume of boiled distilled water and neutralised formaldehyde is used, the initial formol titration of the substrate thereby being ignored. For the determination of solutions containing only amino acids or peptides, a water - formaldehyde blank is used.

INDIRECT METHOD—

The solution under examination is first titrated with alkali to a given pH, formaldehyde (brought to the final pH) is added, and the resulting solution is titrated to an end-point at the same or a different pH. The value of the second titration, usually after subtraction of a blank, is taken as a measure of the amino acids and peptides present. The precise pH to which it is recommended that the preliminary titration should be taken varies. Dunn and Loshakoff[13] recommend pH 6. Neutralisation to pH 7, with litmus or neutral red as indicator, is recommended by Henriques,[14] Henriques and Sorensen,[15,16] Henriques and Gjaldbäk,[17] Luers,[18] Hoppe-Seyler and Thierfelder,[19] Northrop,[20] Van Slyke and Kirk,[4] Levy,[21] Borsook and Dubnoff,[22] Sisco, Cunningham and Kirk[23] and French and Edsall[24]; to pH 8 by Brown[1]; and to pH 9 by Kendall, Day and Walker,[25] Harding and McLean,[26] Berman and Rettger,[27] Cathcart, Paton and Pembrey,[28] Lloyd and Shore,[29] Hawk, Oser and Summerson,[30] Kirk,[31] Mills[32] and Mills, Munro and Leaf.[33]

The final pH to which the titration should be taken after the addition of formaldehyde also varies. Brown[1] recommends pH 8, with phenol red as indicator, and so do Borsook and Dubnoff[22] and Sisco, Cunningham and Kirk,[23] who used electrometric methods. All the other authors mentioned take the titration in formaldehyde to pH 9, with phenolphthalein as indicator. Hence in Figs. 1, 2 and 3 (see p. 490) preliminary titration follows curve B from the pH of solution to pH 6, 7 or 9. Formaldehyde is then added and thereafter the titration follows curve A.

When both preliminary and final titrations are taken to the same pH,[1] the same indicator serves for each and the procedure is referred to as the "one-indicator" technique.[5] When the pH values of the two end-points differ, separate indicators and a "two-indicator" technique are required. To prevent difficulties of colour matching when the second indicator is added, several different devices have been adopted, *viz.*, preliminary neutralisation to pH 7 is carried out with litmus paper,[17] a control colour standard is used,[20] or independent titrations with each of the two indicators are performed on aliquots.[5] Harris[34,35] overcame the difficulty by titrating first to an end-point in alcohol and formaldehyde at pH 9, with phenolphthalein as indicator, and then back-titrating to pH 5 (methyl red) with standard acid. At this latter pH the phenolphthalein used earlier is colourless and does not interfere. The back-titration is taken as a measure of amino acids and peptides.

As with the direct titration, the composition of the blank or control solutions varies. When the absolute amount of amino acids or peptides in a liquid such as urine is to be determined, a water - formaldehyde blank is used. When following the course of proteolysis, Henriques and Gjaldbäk[17] used a sample of the digestion mixture at zero time, but Northrop[20] recommends a water - formaldehyde blank for this purpose. Levy,[21] considering principally the determination of amino acids, found that the best quantitative results are obtained when a blank is omitted. In several of the papers that have been quoted no details of the composition of the blank are given.

PRINCIPLES UNDERLYING APPLICATION OF FORMOL TITRATION—

The reasons for these very great variations of procedure emerge, to some extent, from a combined study of the principles underlying the application of the formol titration and

the historical development of its use. Certain principles that have taken many years to become established, and which are still the source of confusion, may be stated as follows—

(*i*) *Groups determined by direct and indirect titration*—Direct titration measures the total carboxyl groups of amino acids and peptides; indirect titration measures the total amino groups of amino acids, peptides, primary and secondary amines and ammonia.[4,5] This occurs notwithstanding that in both procedures it is the basicity of the $-NH_2$ or $-NH-$ group that is altered by reaction with formaldehyde. This

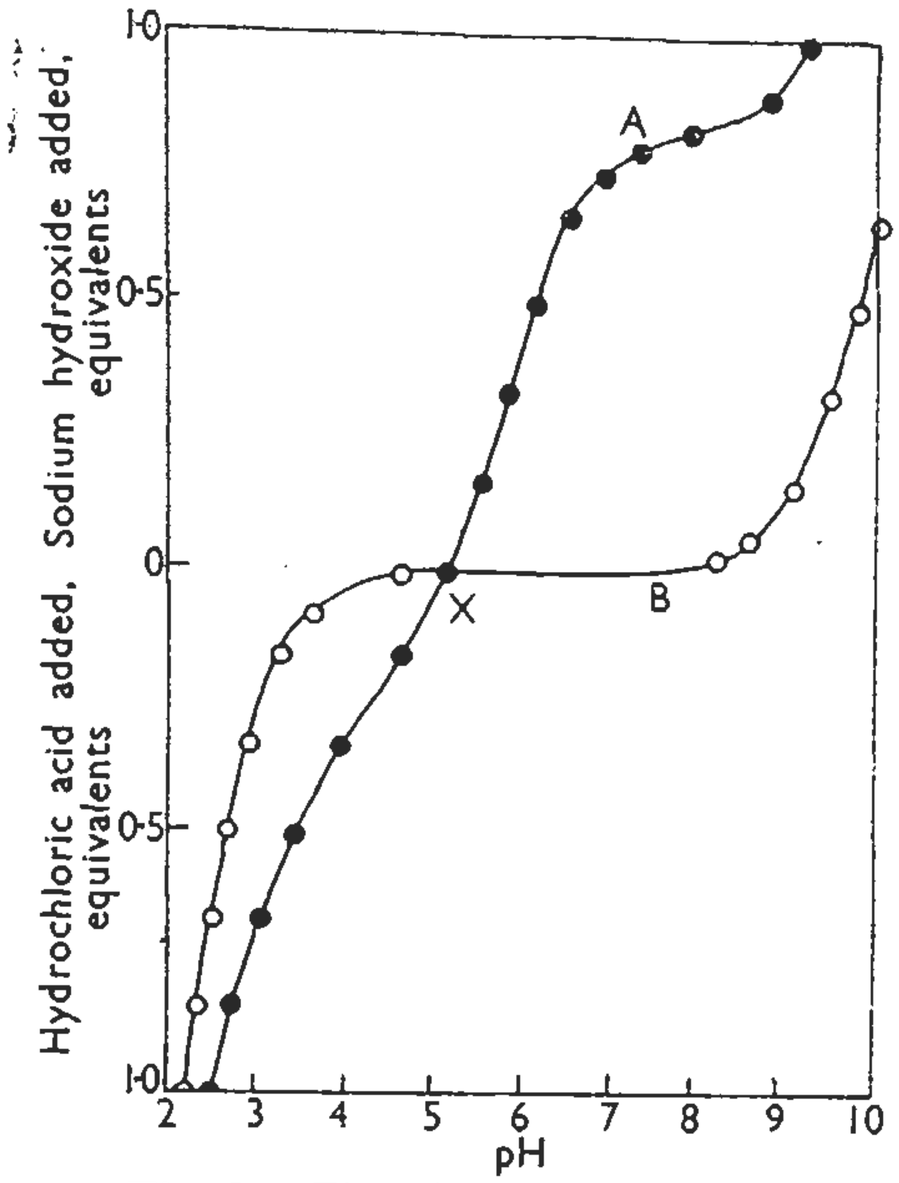

Fig. 1. Titration curves of glycine in 0·04 *M* aqueous solution at room temperature

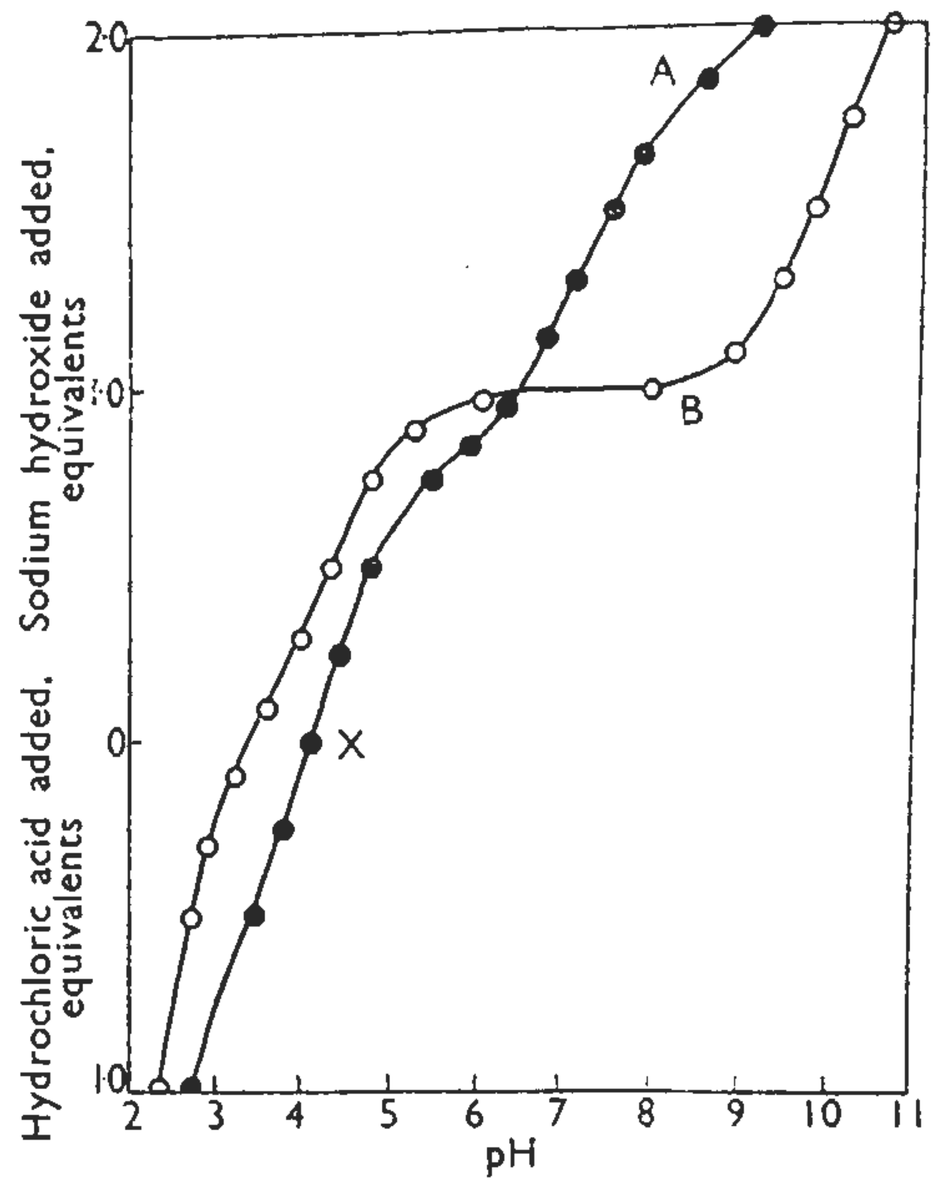

Fig. 2. Titration curves of glutamic acid in 0·04 *M* aqueous solution at room temperature

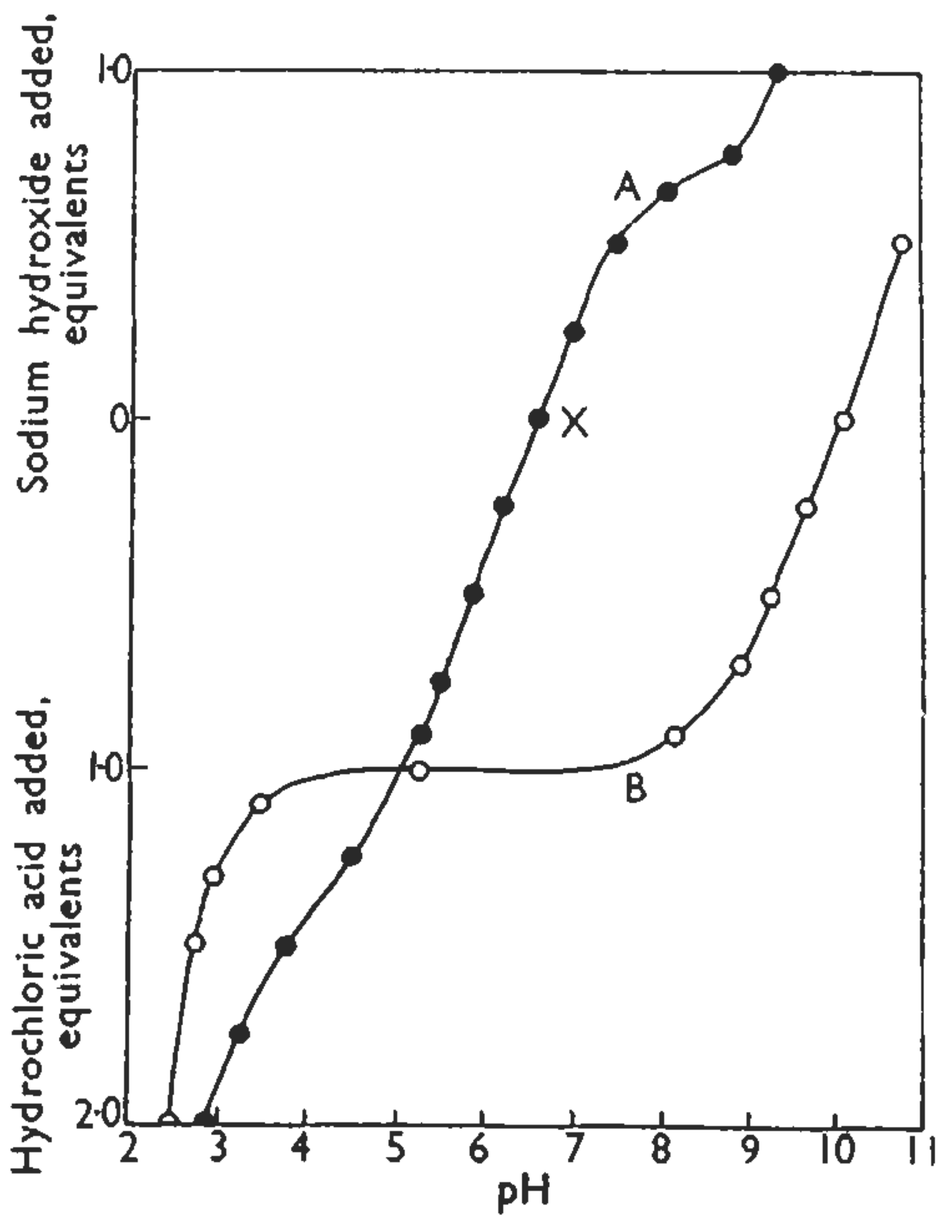

Fig. 3. Titration curves of lysine in 0·04 *M* aqueous solution at room temperature

In Figs. 1, 2 and 3, curve A was obtained with formaldehyde added. X marks the beginning of the direct titration

principle is illustrated by reference to Figs. 1, 2 and 3. Direct titration of a monoaminomonocarboxylic acid such as glycine from its pH of solution to pH 9 in formaldehyde requires one equivalent of base. Indirect titration, after neutralisation to, say, pH 7, similarly requires one equivalent of base. For a dicarboxylic acid such as glutamic acid, direct titration from the pH of solution requires however two equivalents of base, but indirect titration from pH 7 only one. For a diamino acid such as lysine the position is reversed; direct titration requires only one equivalent, indirect titration two.

(*ii*) *Accuracy of direct and indirect titrations*—With solutions containing only amino acids or peptides or both, the direct titration determines total carboxyl groups with almost theoretical accuracy; the indirect titration does not give theoretical accuracy unless the isoelectric point or points of the substance or substances being titrated and the pH of preliminary neutralisation are approximately the same.

The direct titration gives theoretical accuracy because each component amino acid or peptide is effectually titrated from its pH of solution to the chosen end-point in formaldehyde. This is what would be expected when any weak acid such as phosphoric acid or acetic acid or mixtures of both is titrated. With the indirect titration, it will be seen from Figs. 2 and 3 that titration from pH 7 in water to pH 9 in formaldehyde will require nearly the theoretical amounts of base, but that with a one-indicator technique, from, say, pH 8 or 9 in water to pH 8 or 9 in formaldehyde, less than the theoretical amount of base may be needed. Different amino acids may require differing end-points in order to be titrated with optimum accuracy, and this partly accounts for many of the modifications of the indirect procedure that have been devised.

(*iii*) *Presence of other titratable substances*—With solutions such as urine, which contain titratable substances other than amino acids and peptides, both procedures will fail to determine amino or carboxyl groups accurately, unless the interfering substances can be removed, *e.g.*, by filtration of phosphates and carbonates precipitated with barium chloride and hydroxide,[14] or unless allowance can be made for them in controls, or unless the indirect one-indicator technique be used, in which after adding formaldehyde only amino groups will be titrated. For urine and similar solutions the indirect procedure should be used and a choice made between two errors that cannot usually both be eradicated; with a one-indicator technique there is a risk of under-estimation of amino groups, with a two-indicator technique other groups titrating between the chosen pHs are included in the determination.

(*iv*) *Accuracy of direct titration in proteolytic digests*—With proteolytic digests, in which only the increase of amino groups is to be determined, quantitative accuracy may be obtained by the direct method despite the presence of other titratable substances.[2,5] This is because it is possible to use as a blank the digestion-mixture at zero time. Hence, even though digestion takes place at, say, pH 2, liberated amino acids and peptides are in effect titrated from their own pH of solution. On theoretical grounds the indirect titration could also be used to determine the degree of proteolysis, but it is shown experimentally later that, when this is attempted, considerable inaccuracies may arise. Should side-reactions during digestion cause liberation of other titratable groups, these would be determined by the direct method and an error introduced.

(*v*) *Blanks*—It follows that when the degree of proteolysis is being determined, only a digestion mixture at zero time should be used as blank. On other occasions a water - formaldehyde blank is suitable.

(*vi*) *Final concentration of formaldehyde*—The optimal concentration of formaldehyde in the reaction mixture at the end-point should be 6 to 9 per cent.[21]

It should be noted that these principles are derived in the main from theoretical considerations. Practical confirmation of the theoretical behaviour of the direct and indirect procedures has been obtained for single amino acids,[34,35,36] but not for mixtures of amino acids and proteolytic digests, and only occasionally for peptides.

The confusion that has surrounded the use of both procedures has arisen from lack of appreciation of these principles. Sorensen[6] originally used the direct procedure and applied

it first to pure solutions of amino acids, when its quantitative accuracy was established, and then to protein digests. The indirect procedure was devised by Henriques[14] for determination of amino acids in urine. After precipitation of phosphates and carbonates with barium chloride and barium hydroxide, the filtered urine was neutralised to litmus in order to prevent titration of the second carboxyl groups of the dicarboxylic amino acids. A footnote in Henriques' paper makes this clear—

> "Lackmuspapier wird zum Neutralisieren angewandt, weil Lösungen von Aminosäuren, die Monocarbonsäure sind, auf Lackmus neutral reagieren, dasselbe gilt von Aminosäuren die Dicarbonsäuren sind, wenn die eine Carboxylgruppe neutralisiert worden ist."

Henriques and Gjaldbäk[17] next applied the indirect method to the determination of proteolytic hydrolysis. As a result, subsequent workers and writers were justified in inferring the indirect method to be of general application to the determination of amino groups, both absolutely, in biological fluids such as urine, and relatively, as when following proteolysis. The method thus assumed greater importance than the direct procedure, and underwent the many modifications that have been described.

Several attempts to restore the balance between the direct and indirect procedures have been made. As early as 1923, Brown[1] found it necessary to quote extensively from Sorensen's original paper[6] to prove that the direct method was first used—

> ". . . because the description of Sorensen's method in certain text books of physiological chemistry would lead one to believe that the solution to be analysed should be titrated to the colour of phenolphthalein produced in the second stage of the control before the formalin is added."

Jessen-Hansen,[2] in a particularly authoritative account of the formol titration, stressed the importance of the blank and emphasised that, when the degree of proteolytic hydrolysis is to be determined, the indirect procedure is quite unnecessary. Further clarification of the respective roles of the two procedures was achieved by Van Slyke and Kirk[4] and Richardson,[5] arising out of the work of Harris[34,35] and Birch and Harris.[36]

Despite these attempts the quantitative accuracy of the direct titration is still challenged. Hence French and Edsall[24] write—

> ". . . if the titration is begun at the iso-electric point of the amino-acid and carried to an end-point near pH 9 in formaldehyde, the titration gives a measure of the free carboxyl groups. If the titration is started at pH 7 in water and carried to the same end-point, it gives a measure of the free amino-groups present. The latter method is generally the condition of choice; obviously the first can only be applied to a single amino-acid or to a mixture of amino-acids which are all of the same charge type."

These arguments would imply that the direct procedure is quantitatively inaccurate when determining, for example, the degree of proteolysis, unless the pH of digestion happened to be around 7, and are in this respect the reverse of those developed above. The existence of such differences of interpretation serves to emphasise that for proteolytic digests, mixtures of amino acids and for peptides, the behaviour of the two titration procedures has been deduced theoretically and that no experimental assessment of the accuracy of the titrations has been made. Such an assessment is now attempted with particular reference to determining the degree of proteolysis.

Experimental

Formaldehyde—AnalaR formaldehyde solution was neutralised to a pH of approximately 8·5 with 0·1 *N* sodium hydroxide, phenolphthalein being the indicator, and was used in amounts such that the final formaldehyde concentration of the titration mixture was 6 to 9 per cent.[21]

Amino acids and peptides—Amino acids and dipeptides and tripeptides were used in 0·04 *M* solution. Their purity was checked by paper chromatography.

Potentiometric titration—Acid - base titration curves were determined potentiometrically by using a Marconi (type TF 717A) pH meter with a temperature compensator. The titration unit consisted of an open 100-ml Pyrex-glass beaker into which dipped an electric stirrer, a glass electrode (Cambridge Instrument Co. Ltd.) and a pencil-type calomel half-cell modified by Mr. G. E. Newman from a design by Butler.[37] A standard pH of 4·00 at 15° C was given

by an aqueous solution containing 10·211 g of potassium hydrogen phthalate per litre (0·05 *M*). The sleeve of the calomel half-cell was detached and washed before each titration. The protein concentration in the titration vessel did not exceed 400 mg per 100 ml at any time.

Conditions simulating proteolysis—In order to investigate the accuracy of the direct and indirect procedures when the degree of proteolysis was being determined, it was necessary to produce conditions identical with those under which proteolytic digests are titrated, but in which the increase of titratable substances was accurately known. This was achieved by titrating both in water and, after adding formaldehyde, mixtures of buffer (glycine - hydrochloric acid or sodium acetate - hydrochloric acid or potassium dihydrogen phosphate - sodium monohydrogen phosphate) and protein (plasma or serum albumin or casein) and comparing them with the same mixtures to which known amounts of amino acids or peptides had been added. The titration curves of buffer + plasma simulate the buffer + protein + enzyme blanks (digestion at zero time), which should be used in determining the degree of proteolysis, and the buffer + plasma + amino acid or peptide curves simulate those of a buffer + protein + enzyme digest, but with the advantage that the increase of titratable groups is already known.

Results

Preliminary indicator titrations—

Comparison of the direct and indirect procedures was first attempted with indicator titrations under conditions simulating those of protein hydrolysis. In order to explain the results that were obtained, it became necessary to repeat the work with titration curves

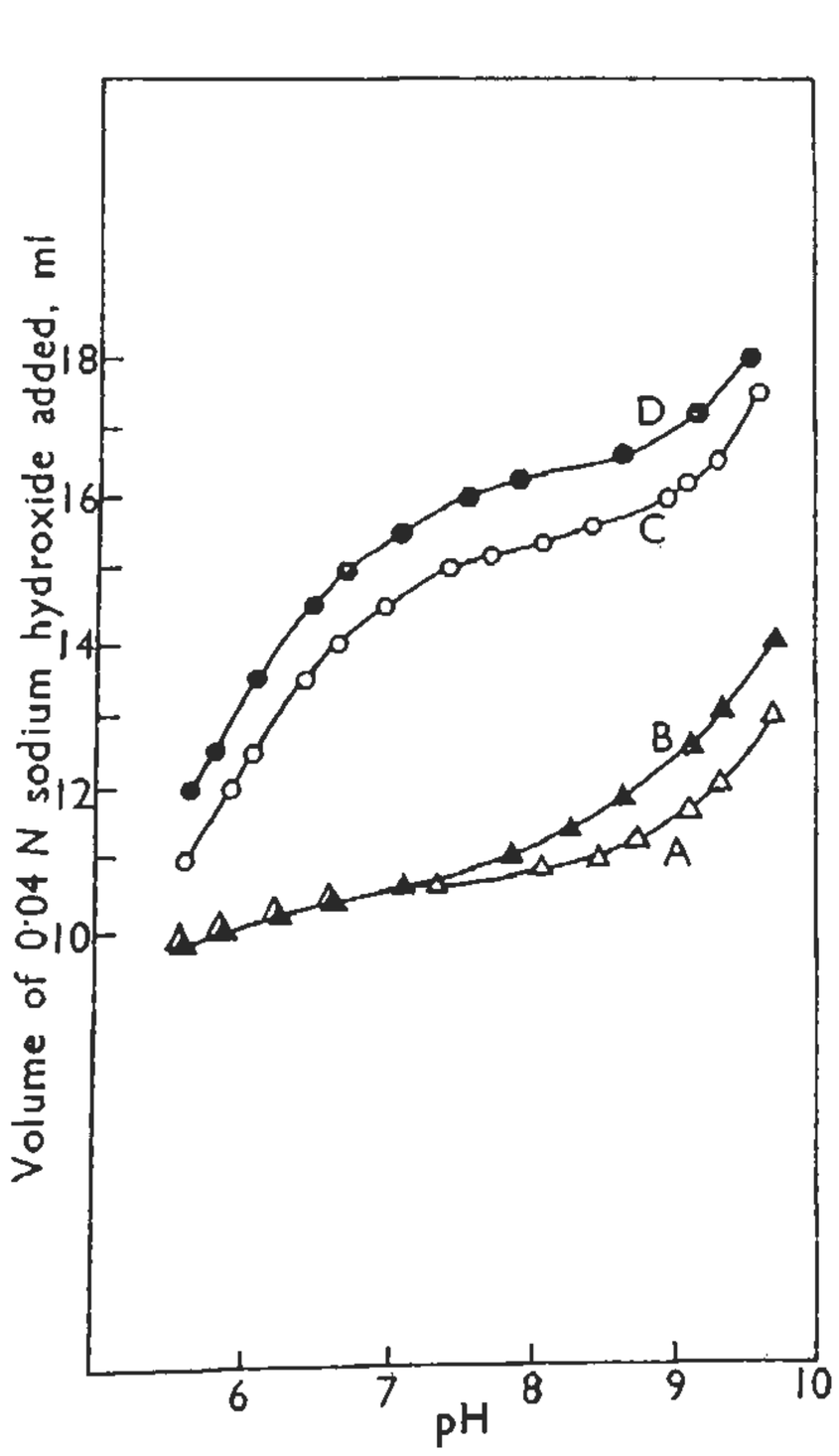

Fig. 4. Formol titration of 1 ml of 0·04 *M* glycylglycine under conditions simulating proteolytic digestion. Buffer: glycine - hydrochloric acid at pH 1·42

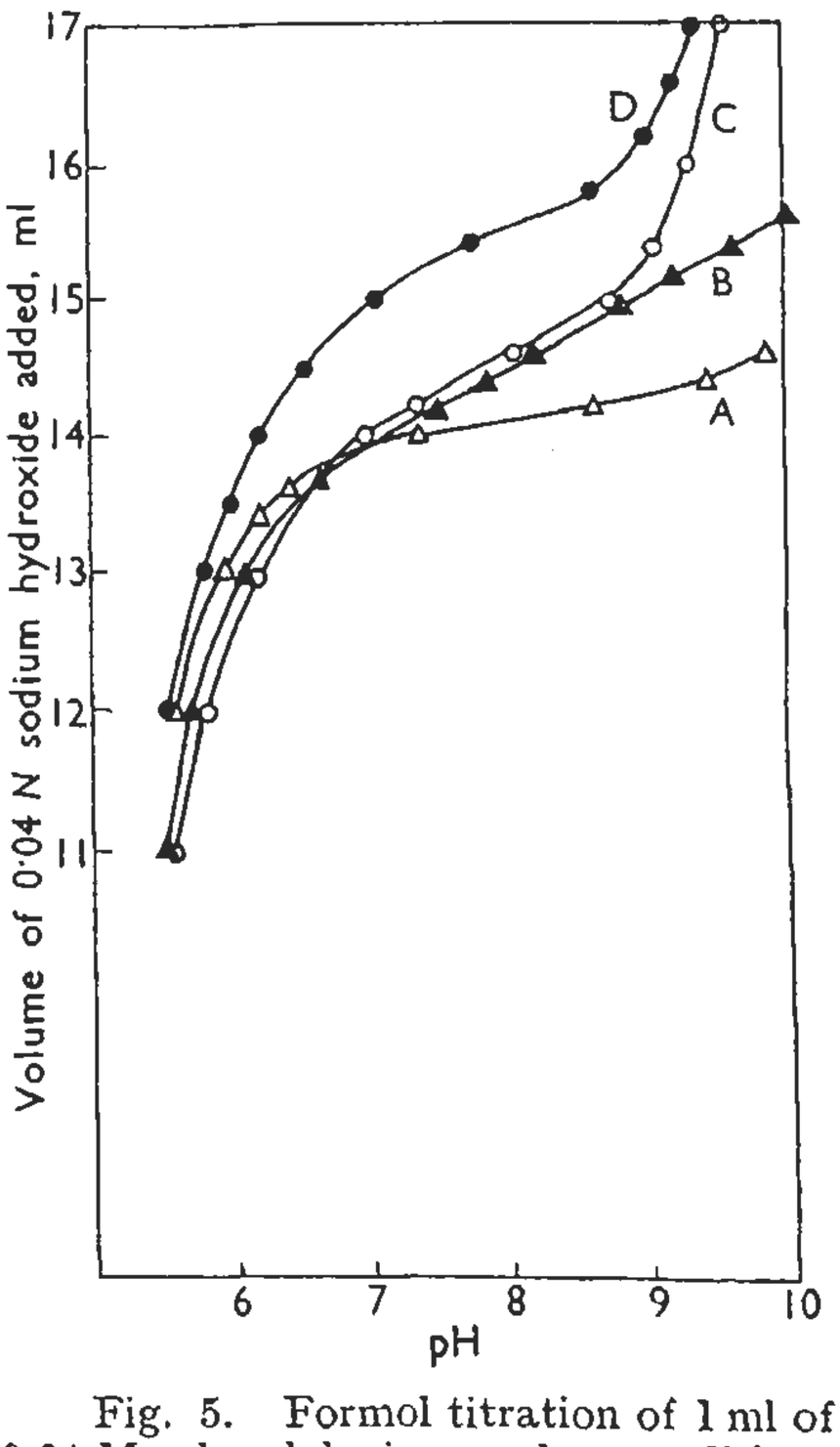

Fig. 5. Formol titration of 1 ml of 0·04 *M* glycylglycine under conditions simulating proteolytic digestion. Buffer: sodium acetate - hydrochloric acid at pH 3·61

In Figs. 4 and 5: curve A, titration of buffer + plasma; curve B, titration of buffer + plasma + glycylglycine; curve C, titration of buffer + plasma + formaldehyde; curve D, titration of buffer + plasma + glycylglycine + formaldehyde.

Direct titration is the difference at the chosen pH between curves D and C. Indirect titration is the difference, less the blank, between curves D and B at the chosen pH. The blank is the difference between curves C and A at the chosen pH

derived potentiometrically and, since these are considered fully later, only the conclusions of the indicator experiments are given, *viz.*—

(*a*) Added amino acids or peptides gave by direct titration, with phenolphthalein as indicator, values that were between 85 and 95 per cent. of the theoretical. Titration to a pale pink colour (pH about 8·5) gave slightly more consistent readings and easier matching than to a deeper colour of approximately pH 9·0. At pH 8·5 the standard deviation for the determination of glycylglycine was 0·01 ml and at pH 9·0 it was 0·02 ml.

(*b*) With the indirect method, the matching of a first end-point at pH 7 to neutral red was difficult in both control and unknown tubes and in duplicates, with consequent lack of reproducibility of results.

(*c*) When pH 9 (phenolphthalein) was used for preliminary neutralisation, very low formol titrations were obtained for peptides, from 0 to 25 per cent. of the theoretical. The titrations for amino acids were higher, but still low, being from 50 to 90 per cent. of the theoretical.

With glycylglycine, for which the theoretical titre should be 1·00 ml, the direct titre to pH 8·5 was 0·90 ml and the indirect titre (*a*) after neutralisation to pH 7 was 0·84 ml and (*b*) after neutralisation to pH 9 was 0·21 ml.

The course of both the direct and indirect procedures was next followed more fully by means of potentiometric titration curves.

POTENTIOMETRIC TITRATIONS—

Figs. 4, 5 and 6 show the potentiometric titration curves with and without formaldehyde of glycylglycine in the presence of plasma and, respectively, of glycine - hydrochloric acid buffer, sodium acetate - hydrochloric acid buffer and phosphate buffer. In each instance

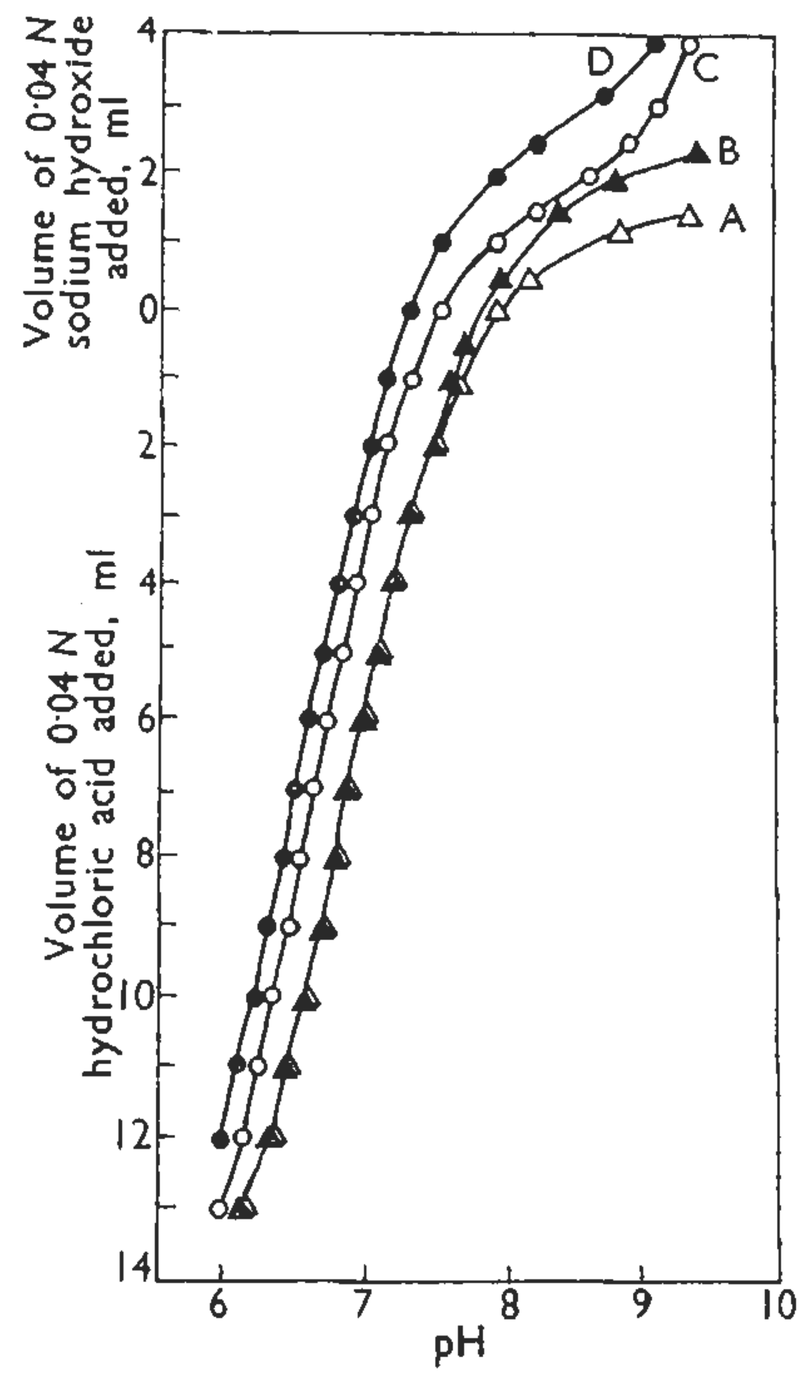

Fig. 6. Formol titration of 1 ml of 0·04 *M* glycylglycine under conditions simulating proteolytic digestion. Buffer: phosphate buffer at pH 8·04 Titration curves as for Figs. 4 and 5

curves with and without formaldehyde are given for plasma plus buffer alone in order to permit the blank titration to be accurately measured. The total volume of fluid upon which each curve has been determined has been kept the same by substituting an equal

volume of distilled water for any missing component. It is possible from these curves to calculate the formol titration by the direct method to either of the final pH values, 8·5 or 9, and by the indirect method by using any of the preliminary pH values 6, 7 or 9, and a final pH of 9. For the latter methods the curves without formaldehyde are followed as far as the selected preliminary pH value, say 7, and then the formaldehyde curves, from a point corresponding to the same amount of added alkali until the end-point of pH 9 is reached. Results calculated from Figs. 4, 5 and 6, and those obtained for several other amino acids and peptides from similar data are recorded in Table I.

Table I

Comparison of formol titration of amino acids, peptides and mixtures by the direct and indirect procedures

Substance taken (1 ml of 0·04 *M* solution)	Buffer and protein mixture		Volume of 0·04 *M* sodium hydroxide required in titration by the direct method to—		Volume of 0·04 *M* sodium hydroxide required in titration by the indirect method, to a final pH of 9·0, from initially—		
			pH 8·5, ml	pH 9·0, ml	pH 6·0, ml	pH 7·0, ml	pH 9·0, ml
Amino acids—							
Glycine	Glycine - HCl	Plasma	0·88	0·94	0·94	0·94	0·78
Alanine	Sodium acetate - HCl	Plasma	0·99	0·96	0·98	0·95	0·81
Leucine	Sodium acetate - HCl	Plasma	1·07	1·05	0·98	1·07	0·90
Tyrosine	Phosphate	Plasma	0·93	0·86	—	—	0·47
Histidine	Glycine - HCl	Plasma	0·91	0·98	1·47	1·05	0·45
Glutamic acid	Glycine - HCl	Plasma	1·99	2·04	1·07	1·07	0·89
Glutamic acid	Phosphate	Plasma	1·89	1·90	—	0·90	0·80
Lysine	Glycine - HCl	Plasma	0·83	0·84	1·73	1·76	1·46
Lysine	Phosphate	Plasma	0·85	0·85	1·80	1·78	1·44
Peptides—							
Glycylglycine	Glycine - HCl	Plasma	0·88	0·87	0·84	0·78	0
Glycylglycine	Sodium acetate - HCl	Plasma	0·94	0·89	1·00	0·89	0·09
Glycylglycine	Phosphate	Plasma	0·86	0·85	—	0·85	0·13
Glycyltyrosine	Phosphate	Plasma	0·86	0·83	—	—	0·24
Glycylglycylglycine	Glycine - HCl	Plasma	1·05	1·07	1·04	0·97	0·14
Glycylglycylglycine	Sodium acetate - HCl	Plasma	0·93	0·95	1·05	0·96	0·16
Glycylglycylglycine	Phosphate	Plasma	0·94	0·94	—	0·90	0·02
Glycylglycylglycine	Phosphate	Serum albumin	1·00	1·05	1·05	0·95	0·17
Glycylglycylglycine	Phosphate	Casein	0·95	0·88	0·93	0·90	0
Leucylglycylglycine	Phosphate	Plasma	0·98	0·93	—	—	0·11
Mixtures—							
Alanine + leucine	Sodium acetate - HCl	Plasma	2·00	1·93	1·89	1·95	1·71
Alanine + lysine	Sodium acetate - HCl	Plasma	1·81	1·76	2·65	2·68	2·22
Leucine + lysine	Sodium acetate - HCl	Plasma	1·88	1·83	—	—	—
Leucine + lysine + alanine	Sodium acetate - HCl	Plasma	2·99	2·92	—	—	—
Glycylglycine + glycyltyrosine + glutamic acid + histidine	Phosphate	Plasma	4·40	4·54	4·30	3·62	1·60
Plasma, peptic digest	Glycine - HCl		0·92	0·86	0·79	0·54	0

Discussion of results

It will be seen from Table I that the direct method, with a buffer - protein - formaldehyde blank and an end-point of pH 8·5, gave results varying from 83 to 107 per cent. of the theoretical for all the amino acids and peptides investigated, except glutamic acid or mixtures containing it. Both carboxyl groups of this amino acid were titrated, in accordance with the predictions of Henriques[14] and Richardson[5] and the data of Van Slyke and Kirk.[4] Preliminary neutralisation to pH 6 or to pH 7 yielded very similar results, except that both amino groups of lysine were now titrated, again in accordance with the data of Van Slyke and Kirk, and that from pH 6 histidine was over-titrated.

The variability of the results obtained by direct titration and their general tendency to fall rather lower than the theoretical values may be explained partly by the error of the titration procedure (in these experiments the standard deviation was 1 per cent., *i.e.*, 0·01 ml of 0·04 *N* sodium hydroxide), partly by the fact that the ideal end-point of the titration varies slightly for individual amino acids and peptides so that the choice of any single pH, such as 8·5 or 9·0, is inevitably a compromise; and partly by the impossibility, on theoretical grounds,[3,5,12,34,35] of ever achieving a fully theoretically quantitative titration. It should be noted also that titration values for the same amino acid or peptide differ in different buffer and protein mixtures more widely than would be expected from the error of titration. The reason for this is not clear and certain possibilities such as complex formation are under investigation.

Despite variability of results caused by the factors discussed above, Table I shows that the direct method, with an end-point at pH 8·5 or 9·0, will determine amino acids and peptides, singly or in mixtures, to within the limits 83 to 107 per cent. of their theoretical value. Whichever of these final pH values is chosen would appear to make little difference to the results obtained by the direct method, the widest deviation being 7 per cent. The titration curves (Figs. 4, 5 and 6), under the conditions of these experiments, begin to rise, however, at about pH 9, so that pH 8·5 is much more nearly at the centre of the more horizontal portion of the curves, at which the pH changes most sharply with increasing volume of added alkali. For indicator titrations, therefore, pH 8·5 (a pale pink to phenolphthalein) would be preferable to pH 9, because of the potentially more sensitive end-point, and this was the finding in the preliminary indicator experiments. Since the pH range of the relatively flat part of the curve is a function primarily of the formaldehyde concentration,[21,36] it is only at the defined formaldehyde concentrations of 6 to 9 per cent. that pH 8·5 gives the more sensitive end-point. With lower final formaldehyde concentrations a higher end-point may be more desirable.

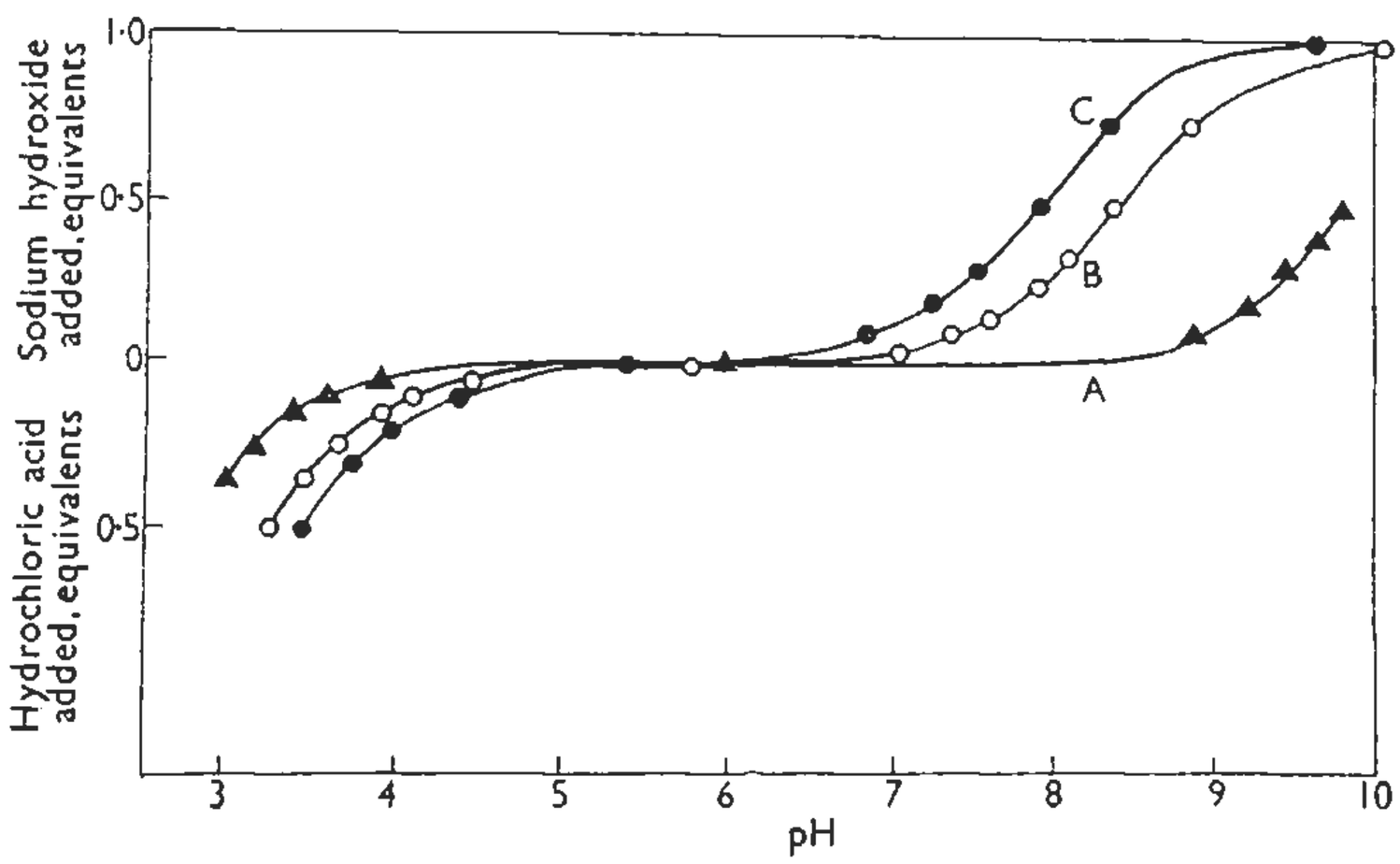

Fig. 7. Titration curves in 0·04 *M* aqueous solution at room temperature: curve A, glycine; curve B, glycylglycine; curve C, glycylglycylglycine

When the indirect techniques are compared, it is observed that the titrations of amino acids from pH 6 and from pH 7 are more or less identical (except for histidine), whereas those of peptides from pH 6 are slightly higher than from pH 7. The titrations from pH 9 are lower both for amino acids and peptides, those for peptides being particularly low. The reason for these findings is seen in Fig. 7. An amino acid such as glycine is partly titrated by the initial adjustment to pH 9 in water, so that after formaldehyde has been added a smaller than theoretical value is obtained. Peptides such as glycylglycine and glycylglycylglycine are, however, almost completely titrated at pH 9 in water, so that when formaldehyde has been added, the additional titration is very small indeed. Even at pH 7 in water, peptides, unlike amino acids, are slightly titrated[34] (see Fig. 7), so that values from pH 7 are usually less than those from pH 6. This is seen particularly clearly with a peptic digest (see Table I), which consists mainly of polypeptides. For this the titration from pH 7 was 0·54 ml and that from pH 6 was 0·79 ml of 0·04 *N* sodium hydroxide. Hence preliminary neutralisation

to pH 9, and for peptides even to pH 7, is clearly undesirable, as it results in a considerable loss of quantitative accuracy. The method still gives a "measure" of proteolytic hydrolysis and will be much more nearly quantitative for digests, such as tryptic, that consist mainly of free amino acids.

Although preliminary neutralisation to pH 6, and for amino acids to pH 7, should yield reasonably good results, it was found in the indicator titrations that the method gives poor reproducibility. The reason for this is revealed by the titration curves (Figs. 4, 5 and 6). Each final value is determined as the resultant of four indicator end-points, two for the digest and two for the blank, and none of these end-points, at pH 6, or 7 and 9 in the presence of buffer and protein, is sharp.

Despite the fact that titration commenced at widely differing pH values in the three buffers that were used, the results obtained by direct potentiometric titration appear to be as closely quantitative as those obtained by any of the indirect procedures. Nor were the values obtained with the most acid buffer (glycine - hydrochloric acid) higher than those obtained with the alkaline phosphate buffer. Further, mixtures of amino acids and peptides not "of the same charge type" were found to be titrated as nearly quantitatively as the sum of their individual components (see Table I). It would seem therefore that, despite arguments such as those of French and Edsall,[24] the direct method can in fact be used for the measurement of amino-acid nitrogen in a mixture with the same degree of quantitative accuracy as for the single pure components thereof and at least as accurately as with any indirect procedure. The theoretical principles stated earlier are thus validated.

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The Determination of Small Amounts of *o*-Phenylphenol

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Methods are described for the extraction and determination of the *o*-phenylphenol added as a fungicide to various materials. The determinations are made by ultra-violet spectrophotometry, colorimetry or fluorescence. The ultra-violet and colorimetric methods are rapid, sensitive and reasonably specific. In the colorimetric method use is made of a commercially available stabilised diazo compound, Brentamine fast red GG; it has advantages over the more usual colorimetric methods for phenols in rapidity and specificity. Both methods give satisfactory results with a variety of treated materials.

The fluorescence method, although specific and sensitive, is subject to serious interference and is of use mainly as a qualitative test.

o-PHENYLPHENOL is finding increasing use as a fungicide for diverse materials and the need has arisen for methods of determination that will be adequate at the rather low concentrations employed.

Various colorimetric methods have been proposed.[1,2,3,4] Tomkin and Isherwood[3] employed a method in which, after separation by steam-distillation, followed by extractions with light petroleum and sodium hydroxide solution, the *o*-phenylphenol was finally coupled with diazotised sulphanilic acid. Gottlieb and Marsh[4] made use of a reaction common to phenols unsubstituted in the *para* position, in which a colour is produced with 4-amino-antipyrine in the presence of potassium ferricyanide and sodium carbonate. However, we have found these methods lacking in either simplicity, specificity or rapidity.

Cox[5] suggested making use of ultra-violet spectroscopy and also mentioned the possibility of utilising the strong fluorescence given by *o*-phenylphenol in ethanolic sodium hydroxide solution. We have examined his suggestions in some detail, and in addition have used a stabilised diazo compound, Brentamine fast red GG, as a new reagent in a rapid and sensitive colorimetric method.

PRELIMINARY TREATMENT OF SAMPLES

The pre-treatment of the sample depends upon the nature of the material and some typical methods are described below. The quantity of sample taken will naturally be determined by the concentration of *o*-phenylphenol present and by the amount necessary to secure a representative portion. The sample weights given should be regarded as a guide only and the final solutions, however prepared, must be adjusted to contain between 5 and 25 μg of *o*-phenylphenol per ml.

"DISINFECTANT SALT" (*i.e.*, COMMERCIAL SODIUM CHLORIDE PLUS ADDED FUNGICIDE)—

Dissolve 1 g of sample in 50 ml of water. Add 10 ml of *M* sodium hydroxide solution to ensure complete solution of the *o*-phenylphenol and dilute to a suitable volume (say, 100 ml) with water. No interference has been encountered in the determination of *o*-phenylphenol in this material by any of the three methods to be described.

DISINFECTANT FORMULATIONS—

Dissolve 5 g of sample in spectroscopic-quality *cyclo*hexane and dilute to 100 ml. If the sample is aqueous, acidify it and extract the solution with five 10-ml portions of *cyclo*hexane. A spectrophotometric determination of the *o*-phenylphenol may then suffice, although if interference occurs or is suspected (see p. 500) it will be necessary to extract the *o*-phenylphenol from the *cyclo*hexane solution with sodium hydroxide solution.