A simple method of titration with a cationic detergent has been developed which permits rapid estimation of the concentration of urinary protein with a minimum of manipulations and time. The method depends on the formation of an insoluble anion-cation complex between quaternary ammonium ions, hereinafter referred to as the cationic detergent, and proteins, in the present instance mainly human albumin, at alkaline pH. No protein is precipitated from solution by the addition of the cationic detergent until a slight excess of this is present, at which time the solution shows faint but definite turbidity. Addition of more cationic detergent results at first in increasing turbidity, but as the addition is continued the turbidity decreases, almost as abruptly as it appeared, to give a nearly clear solution. Further addition of the cationic detergent is not attended by reappearance of turbidity. The end-point used in the present method is the appearance of a definite turbidity. The protein-quaternary ammonium ion complex formation is sensitive to ionic strength, non-electrolyte concentration, pH, and type of protein present (see, for example, Polonovski and Macheboeuf (1), Schmidt (2), and Valko (3)). For this reason, the method is restricted for accurate results to urine protein of the order of 2 or more gm. per liter (0.4 or more mg. of protein per sample) in order that the dilutions made may be sufficient to reduce the factors of urine ionic strength, non-electrolytes, and pH to negligible proportions. The method is, however, applicable to most cases of significant proteinuria. The simplicity of the procedure is indicated by the fact that we have been able to complete nearly 200 analyses in an afternoon.

If the end-point is determined by the optical density change in a photometer, the method can be used for samples containing as little as 0.02 mg. of protein.

* The preliminary phases of this work were started while the author was a Fellow in the Medical Sciences of the National Research Council in 1945-46.
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Reagents

Alkyldimethylbenzylammonium chloride,1 0.1 per cent solution in distilled water. The solutions have been found to be stable for at least 3 months. This solution will be referred to as "the detergent."

2 N NaOH.

Procedure

Preparation of Standard Curve Relating Amount of Added Detergent to Amount of Protein in Sample—Because of the uncertain composition of the detergents available it is essential that a standard curve be prepared relating the amount of added detergent to the amount of protein in the sample. For the present work a series of twelve urine samples was selected for use as a standard. The protein concentration of the urines was calculated from the difference between total nitrogen and nitrogen after precipitation of the proteins with an equal volume of 10 per cent trichloroacetic acid. The technique used for digestion and distillation was that described by Hiller, Plazin, and Van Slyke (4). Turbidity titrations were then carried out as described below; the results were plotted with the protein concentration as ordinates and the ml. of detergent used as abscissae. An example of a standard curve with human serum albumin is given in Fig. 1.

Titration of Urine Proteins—0.1 or 0.2 ml. of the urine samples containing 0.4 or more mg. of protein is accurately pipetted into 2.5 X 10 cm. test-tubes, and 4 ml. of distilled water are added, followed by 0.2 ml. of 2 N NaOH. (The pH of the solution is approximately 13.) The detergent is then added from a 2 ml. burette, with swirling, until the appearance of a definite and permanent turbidity. This turbidity is most easily detected by titrating against a black background and by having a beam of light passing through the test-tube at right angles. Reproducibility may be insured by comparing with samples of the standards titrated previously, but after a few preliminary trials it will be found that the reproducibility of this

1 Unless otherwise specified, the results reported here have been obtained with myristamidopropyl dimethylbenzylammonium chloride (trade name, Aerosol M), manufactured by the American Cyanamid Company, 30 Rockefeller Plaza, New York 20. For use 1 ml. of the concentrated detergent is diluted to 500 ml. in distilled water. We are indebted to the American Cyanamid Company for several samples of their product. Equally satisfactory and similar results have been obtained with cetyl dimethylbenzylammonium chloride and dodecyl dimethylbenzylammonium chloride in 0.1 per cent solutions. These last two compounds are available from the Onyx Oil and Chemical Company, Jersey City 2, New Jersey. A mixture of alkyl dimethylbenzylammonium chlorides (alkyl = C11H23 to C18H37), manufactured by the Winthrop Chemical Company, Inc., 170 Varick Street, New York 13, under the trade name Zephiran, may also be used in 0.1 per cent concentration, though it is slightly less satisfactory.
turbidity end-point is no more difficult than in ordinary acid-base titrations with phenolphthalein as an indicator. The mg. of protein present in the sample analyzed are then calculated from a standard curve, established from urines containing varying amounts of protein calculated from Kjeldahl N determinations.

Calculations—A standard curve relating ml. of detergent to protein concentration is prepared as described above. The results for unknowns are read off the curve and the proper correction made for dilution of the urine.

Results

Standard Curve with Human Serum Albumin; Reproducibility of Results—Fig. 1 shows the relationship of the ml. of detergent required to reach the end-point and the mg. of human serum albumin present as determined by the Kjeldahl method. An average of six titrations was carried out for each point of the curve. The ml. of detergent added and the estimated standard deviations for each point were 0.519 ± 0.023, 0.863 ± 0.012, 1.220 ± 0.019, 1.528 ± 0.011, 1.834 ± 0.020, 2.224 ± 0.022. The dotted line in Fig. 1 (the extension of the straight line through the points) shows that the true end-point was overstepped by an absolute number of ml. for each point on the curve. The overstepping is equal to the X intercept of the extended straight line. This overstepping, equivalent to 0.18 ml. of detergent solution, is due to the fact that the first appearance of turbidity was not taken as the end-point, but rather the appearance of a definite turbidity, the reproducibility of which is indicated by the standard deviations above.

Comparison of Results Obtained by Turbidity Titration with Several Detergents by Biuret Reaction—In Fig. 2 are plotted the protein concentrations of urine of patients with the nephrotic syndrome, some of whom were receiving large amounts of human serum albumin intravenously. The results obtained by turbidity titration with myristamidopropyl dimethylbenzylammonium chloride are plotted as ordinates, while the results obtained by the biuret reaction (5) and the Kjeldahl method (as described above under "Preparation of standard curve") are plotted as abscissae. Twelve Kjeldahl determinations of urine protein were used to calculate the equivalence of ml. of detergent and mg. of urinary protein. The estimated standard deviation of results by the turbidity titration from these by the Kjeldahl method was ±3.71 per cent. When the turbidity titration results were calculated as per cent of the biuret results, the mean for the 105 estimations was 100.5 per cent, with an estimated standard deviation of ±4.8 per cent.

In another smaller series of eleven urines the results of turbidity titrations with other alkyl dimethyl benzyl ammonium compounds were compared with results of the biuret reaction as applied by Hiller, Greif, and Beckman (5).
For myristamidopropyldimethylbenzylammonium chloride the mean was 100.9 per cent of the biuret results, with an estimated standard deviation of ±4.45 per cent; for cetyldimethylbenzylammonium chloride the mean was 100.6 per cent of the biuret results, with an estimated standard deviation of ±3.65 per cent; for dodecyldimethylbenzylammonium chloride the mean was 99.6 per cent of the biuret results, with an estimated standard deviation of ±4.59 per cent; and for Zephiran the mean was 100.9 per cent of the biuret results, with an estimated standard deviation of ±5.56.

Fig. 1. "Standard curve" of human serum albumin titrated with myristamidopropyldimethylbenzylammonium chloride (1:500).

Relation of Optical Density to Amounts of Detergent Added to Solutions of Albumin—Fig. 3 shows the result of adding increasing amounts of detergent solution to 0.2, 0.5, 1.0, and 1.5 mg. of human serum albumin under the
conditions described under "Procedure." The solutions were not made up to the same final volume, as it was desired to check the end-point used under the conditions of the estimations. The optical densities were measured in cylindrical cuvettes (12 × 75 mm. outside diameter) in a Coleman model 6 clinical spectrophotometer at \( \lambda = 450 \) m\( \mu \), within about 30 minutes of the addition of detergent to the first of a series. It will be seen that if an optical density of 0.025 is taken as the end-point (this is the approximate optical density used as the visual end-point for the data in Figs. 1 and 2) the number of ml. of detergent added to the sample containing the smallest amount of protein is not in proportion to the number of ml. added to

Fig. 2. Comparison of results obtained by turbidity titration with myristamidopropyl dimethyl benzylammonium chloride (1:500) by the biuret reaction and the Kjeldahl method.
samples containing larger amounts of proteins. Therefore, if protein were calculated as directly proportional to the detergent added, one would somewhat overestimate the amount of protein present, especially for very small amounts of protein. That the estimated "equivalence" end-points are in reasonably good proportion (0.13, 0.30, 0.60, 0.90 ml. of detergent for 0.2, 0.5, 1.0, and 1.5 mg. of protein, respectively), however, confirms the stoichiometry of the procedure already demonstrated in Fig. 1.

![Fig. 3. Relation of optical density to amounts of detergent (myristamidopropyl-dimethylbenzylammonium chloride) added to solutions of human serum albumin. The arrows indicate the estimated "equivalence" end-points.](http://www.jbc.org/)

**Effect of pH**—Fig. 4 shows the optical density curves obtained by adding varying amounts of detergent to the same amount of protein, namely 1.0 mg. of human serum albumin, at pH 7.0, pH 7.8, pH 9.2, respectively, under the conditions described under "Procedure" (pH approximately 13). Addition of 1 ml. of 2N NaOH instead of 0.2 ml. results in a very slight shift of the curve to the right.

**Other Detergents**—A few other cationic detergents have been tried, as
mentioned in foot-note 1. Dimethylbenzylcetylammonium chloride, di-
methylbenzyldodecylammonium chloride, and Zephiran give results sim-
ilar to those obtained with the detergent routinely used as described
above. The high molecular weight alkylimidazolinium compounds are not
satisfactory for the present purposes because of the slowness of the devel-
opment of the turbidity. Alkyltrimethylammonium compounds give
indefinite end-points. Unsatisfactory results were also obtained with an N-

![Graph](http://www.jbc.org/)

*Fig. 4. Effect of pH on titration curves of human serum albumin with myristami-
dopropyl(dimethylbenzyl)ammonium chloride, and with cetylpyridinium
chloride.*

(acylalaminoformylmethyl)pyridinium chloride, and with cetylpyridinium
chloride.

*Relation of Optical Density to Amount of Detergent Added to Plasmas, with
Differing Albumin and Globulin Concentrations*—The method is not at
present recommended for plasma proteins, because they present some
difficulties not encountered in urine. Because of the turbidity present
before titration in some plasmas it is not possible in all cases to use the
visual end-point described above to estimate total protein in plasma. In
some preliminary experiments with plasmas of varying albumin and globulin
concentrations the optical density curves show good correspondence
between ml. of detergent added to reach the "equivalence" end-point and
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Peculiarities appear in the titration curves in the presence of abnormally large amounts of γ-globulins. Further work on the plasma proteins is under way.

DISCUSSION

While a considerable amount of work has been done on anionic and cationic detergent-protein complex formation, the exact nature of the process is not completely known. It is apparent from the work of Putnam and Neurath (6) that the cationic groups of proteins play a major rôle with anionic detergents at acid pH. Similarly at alkaline pH the negative charges of the protein molecules must play a major rôle with cationic detergents. It may be noted, however, that the detergents are protein denaturants (7) and under certain conditions act to accelerate protein hydrolysis (8). Further, there is probably an unfolding of certain proteins in the presence of increasing amounts of detergents; native proteins cannot always be recovered quantitatively after exposure to the detergents (6, 9).

For the present work, a tentative working hypothesis (an extension of that of Polonovski and Macheboeuf (1)) is as follows: At pH 13, the carboxyl, phenolic, and sulfhydryl groups are all dissociated; there are still some positively charged guanidine groups, but the charges of the protein molecule are mainly negative. As detergent is added to the protein solution, the positively charged quaternary ammonium ions are attracted to the negative charges of the protein molecule by Coulomb forces, van der Waals forces playing a rôle dependent on the substituents of the nitrogen of the detergent. As more and more of the negative charges of the protein are “neutralized” by the positively charged quaternary ammonium ions, the protein molecule becomes less and less polar until finally, at the so called “equivalence” end-point, aggregation of the protein molecules becomes possible through van der Waals forces. With further addition of detergent the non-polar portions of the added detergent become associated through van der Waals forces with the non-polar portions of the detergent ions attached to the protein, the protein-detergent complexes become positively charged and repel each other, and dispersal of the aggregates then takes place. The N substituents in the detergent ions play a rôle in determining the extent of the van der Waals forces and may be responsible for the unsatisfactory end-points obtained with the alkyltrimethyl, alkylidimethyl-ethyl, alkylimidazolinium, and alkylpyridinium quaternary compounds. With the alkylidimethylbenzyl compounds the number of moles of detergent required to reach the “equivalence” end-point (Fig. 3) per mole of human serum albumin varies with the detergent used, though it is of the order of magnitude of the sum of the total free carboxyl, phenolic, and sulfhydryl groups calculated from Brand’s data (10). Chain length of the alkyl
group may be of importance in this. A similarity to the zone phenomenon of antigen-antibody titration is evident in the fact that a definite protein-detergent ratio is required to produce aggregation.

In any case, whatever the exact nature of the protein-detergent complex, it is evident that reasonably quantitative results are obtainable by the procedure herein described. A method, to which the present one is similar in principle, was recently introduced by Lambert (11) for the volumetric analysis of anionic and cationic detergents by turbidity titration. Maximum turbidity measured photometrically is used by Lambert as the endpoint, rather than the appearance of a definite turbidity estimated visually. Use of maximum turbidity is possible for the estimation of proteins, but the additional equipment required and the extra time and manipulations involved would reduce the advantages of the present procedure. The technique described by Lambert takes about 5 times as long.

SUMMARY

A rapid simple method for the estimation of urinary protein concentrations is presented. The method depends on the formation of an insoluble complex between quaternary ammonium ions and proteins at pH 13. Endpoints are estimated visually by the appearance of a faint but definite and permanent turbidity. The standard deviation was ±3.71 per cent from determinations by the Kjeldahl method in a series of twelve estimations. In a series of 105 estimations, the standard deviation was ±4.8 per cent from determinations by the biuret reaction. Several types of quaternary ammonium compounds have been tried; the most satisfactory for the present purposes are the alkyl dimethylbenzylammonium group. A brief discussion of the possible mechanism of the quaternary ammonium ion-protein anion complex formation is given.

It is a pleasure to acknowledge the help given by Dr. Hiller and Dr. Greif in making available their biuret procedure before its publication.

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INTERACTIONS OF QUATERNARY AMMONIUM COMPOUNDS AND PROTEINS: A SIMPLE METHOD FOR THE RAPID ESTIMATION OF URINARY PROTEIN CONCENTRATIONS WITH ALKYLDIMETHYLBENZYL-AMMONIUM COMPOUNDS
Francis P. Chinard and With the technical assistance of Dora M. Newell


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