The Isolation and Estimation of Urinary Mucoproteins

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(Received 19 July 1954)

The presence in certain pathological urines of protein-like material which is not coagulated by heat has been realized for some years. It has been called mucin, mucoid, nucleoprotein, phosphoprotein, mucoprotein and nuclealbumin by different investigators and has usually been detected by the turbidity produced on rendering the diluted urine acid with acetic acid. The isolation and characterization of a similar substance in normal urine was first reported by Tamm & Horsfall (1950, 1952), who employed two methods of isolation, one relying on ethanol precipitation and the other on salting-out with 0.58M sodium chloride. Yields in both cases were of the order of 2–2.5 mg./100 ml. of urine. Data on this substance suggest that the term 'mucoprotein' is appropriate (Tamm & Horsfall, 1950, 1952; Gottschalk, 1952; Odin, 1952; Waldron, 1952*a, b*).

The first part of the present paper deals with a method of isolating mucoproteins from normal urine by adsorption on benzoic acid, a technique suggested by its use in the concentration of the urinary and serum gonadotrophins (Katzman & Doisy, 1932; Rimington & Rowlands, 1941). The second part describes a method of estimation employing diphenylamine. This was first used by Jolles (1910) for the demonstration of fructose and later by a number of workers for the estimation of inulin (Corcoran & Pape, 1939; Alving, Rubin & Miller, 1939). More recently it was shown to give a purple colour in acid solution with a serum constituent thought to be mucoprotein (Niazi & State, 1948; Ayala, Moore & Hess, 1951) and with the mucoprotein fetuin from foetal calf serum (Deutsch, 1954). The mucoprotein fraction which we have isolated also gave a purple colour with the acid diphenylamine reagent which proved suitable for quantitative application.

Preliminary reports of this work have been published (Anderson, 1954; Anderson & MacLagan, 1954).

METHODS AND MATERIALS

Nitrogen was determined by the micro-Kjeldahl method in a Markham still (Markham, 1942), reducing sugar by the method of Hagedorn & Jensen (1923), acetyl content by the method of Kuhn & Roth (1933), and hexosamine by the method of Tracey (1952).

Paper chromatography was carried out for amino acids as described by Consden, Gordon & Martin (1944), for sugars as described by Jermyn & Isherwood (1949), and for amino sugars as described by Partridge (1948).

The method of Durrum (1950) and Flynn & de Mayo (1951) was used for paper electrophoresis.

Diphenylamine reagent. Diphenylamine (1 g.) dissolved in a mixture of 90 ml. of glacial acetic acid and 10 ml. of conc. H_2SO_4 , all reagents being AnalaR.

Reaction tubes. The special glass-stoppered tubes ($6 \times \frac{1}{2}$ in.) used for the incubation with the diphenylamine reagent had a vertical groove in the glass stopper which permitted equilibration of pressure with the atmosphere, but allowed mixing by inversion when turned to the closed position.

EXPERIMENTAL

Isolation of mucoprotein A₁. Method 1. To each litre of filtered normal male urine was added, slowly and with constant stirring, 200 ml. of an acetone solution of benzoic acid (20%, w/v). After 2 hr. standing at room temperature with occasional stirring, the precipitate of benzoic acid was filtered off and dissolved in 100 ml. of acetone. After centrifuging, the supernatant fluid was discarded and the residue washed with a further 60 ml. of acetone. It was then treated with 40 ml. of water previously adjusted to pH 9–9.5 with 2N-NaOH. After centrifuging, the supernatant fluid was decanted and the residue washed with a further 40 ml. of water. The combined supernatant fluids were then dialysed against distilled water (2 l.) overnight at room temperature. The dialysed solution was made 0.2M with respect to NaCl by the addition of an aqueous solution of 7% NaCl (w/v) and the mucoprotein precipitated by pouring the solution slowly and with constant stirring into 5 vol. acetone. After complete flocculation of the precipitate (about 1 hr.) it was centrifuged down, washed twice with acetone, dried over $CaCl_2$ *in vacuo* and weighed. 56 l. of urine yielded 4.43 g. of a brown powder. This was dissolved in water and dialysed against running dist. water at room temperature until a constant N content had been reached (35 hr.). The mucoprotein was then reprecipitated by NaCl and acetone. 2.17 g. of a light brown powder was obtained, a yield of 3.87 mg./100 ml. of urine.

Although this yield was higher than those previously reported it did not include the whole of the mucoprotein present, as a further fraction (3.1 mg./100 ml.) was obtained from the supernatant urine, after benzoic acid adsorption, on the addition of 5 vol. ethanol. This fraction contained more reducing substance (30.7%) and considerably less N (5.1%) than that obtained by benzoic acid adsorption.

Isolation of mucoprotein A₂. Method 2. A slightly modified method of isolating mucoprotein A₁ was used, which did not depend on the presence of acetone.

To each litre of filtered male urine was added 125 ml. of 2M sodium benzoate followed by 100 ml. of 2N-HCl at room temperature. The precipitated benzoic acid was filtered off and dissolved in 100 ml. of acetone. After flocculation of the resulting residue (about 30 min.) it was centrifuged down and washed once with acetone. The residue was then treated with 50 ml. of water at pH 9-9.5, the supernatant retained after centrifuging and the residue washed with a further quantity of water. After centrifuging, the combined supernatant fluids were made 0.2M with respect to NaCl as above and the solution poured into 5 vol. acetone. After flocculation the precipitate was centrifuged down, washed twice with acetone and dried under vacuum. 52 l. of urine yielded 7.48 g. of a brown powder. This was dissolved in water and dialysed against running distilled water at room temperature for 120 hr. The small residue that formed during the dialysis was centrifuged down and rejected and the mucoprotein in the supernatant fluid reprecipitated with sodium chloride and acetone as described above. 1.37 g. of a light brown powder was obtained, a yield of 2.6 mg./100 ml. of urine.

Properties. Both mucoproteins A_1 and A_2 dissolved fairly readily in water at pH 7, solution being faster in water previously adjusted to pH 9-9.5 by the addition of 2N-NaOH. No precipitate occurred on adding equal volumes of the following reagents to a 2% (w/v) aqueous solution of the two mucoproteins: trichloroacetic acid (20%), sulphosalicylic acid (10%), picric acid (saturated) and phosphotungstic acid (20%). Precipitation did occur, however, with

5% phosphotungstic acid in 2N-HCl, and with 75% acetone (v/v). In the latter case, flocculation was greatly increased in the presence of 0.2M-NaCl. Both mucoproteins gave positive Molisch, biuret, ninhydrin, xanthoproteic, Millon and Sakaguchi reactions. They gave a purple colour with the diphenylamine reagent. They also gave a colour reaction for *N*-acetylhexosamine with Ehrlich's aldehyde reagent (Morgan & Elson, 1934) on heating with 0.05N- Na_2CO_3 . The substance giving the reaction was dialysable through cellophan. The maximum amount of reducing sugar calculated as glucose, released in 1 hr. (Fig. 1) on hydrolysis with 2N-HCl at 100°, was 17.5%. Results of chemical analysis are given in Table 1.

When subjected to paper electrophoresis at pH 8.6, both mucoproteins, which stained rather faintly with azocarmine, were found to migrate as a rather diffuse band with a mean mobility between that of serum α_1 - and α_2 -globulin. The poor definition of the bands suggested that the preparations were probably not homogeneous.

Chromatographic examination of the hydrolysed mucoproteins

Amino acids. Hydrolysed material (4N-HCl, 100°, 10 hr.) was examined by two-dimensional chromatography, and the following amino acids were identified: aspartic acid, glutamic acid, phenylalanine, tyrosine, valine, proline, arginine, lysine, alanine, threonine, glycine, serine and leucine (isoleucine). In addition, three unknown ninhydrin-reacting spots were encountered. Their positions in the case of A_2 are shown in Fig. 2. Preparation A_1 gave identical results.

Sugars. Hydrolysed material ($\text{N-H}_2\text{SO}_4$, 100°, 6 hr.) was run in ethylacetate-pyridine-water (2:2:1, by vol.), employing the descending technique. On spraying with aniline hydrogen phthalate reagent, galactose, mannose and fucose were detected, the relative quantities of each decreasing in the order given.

Amino sugars. On spraying the amino acid chromatograms with ninhydrin, a large purple-grey spot appeared in addition to those due to the amino acids. This spot was in the position corresponding to that of glucosamine or galactosamine but further identification was not successful.

Fig. 1. Hydrolysis of mucoprotein A_1 with 2N-HCl at 100° as determined by reducing power expressed as glucose.

Table 1. Chemical analysis of mucoproteins A_1 and A_2

	Mucoprotein A_1	Mucoprotein A_2
Nitrogen (%)	10.5	9.5
Reducing activity, expressed as glucose:		
(a) Before hydrolysis	7.2	8.3
(b) Maximum liberated after hydrolysis (2N-HCl, 100°)	17.5	17.5
Acetyl (%)	3.7	4.3
Hexosamine (%)	14.4	16.9

Fig. 2. Two-dimensional chromatogram of the products of mucoprotein A_2 obtained after hydrolysis with 4N-HCl for 10 hr.

THE ESTIMATION OF URINARY MUCOPROTEINS

Specificity. The specificity of the diphenylamine reaction was first investigated. Table 2 shows the colour developed by the substances tested on heating with the diphenylamine reagent for 30 min. at 100°. The chromogenic power of the same amount of urinary mucoprotein is set at 100. It will be seen that the only substances other than mucoprotein found to give strongly positive reactions were fructose and fructose containing compounds. The method of estimating the mucoprotein, however, was devised so as to remove fructose.

Heating conditions. It was found that time of heating was an important factor in the colour intensity produced. A plot of colour intensity (expressed as the colorimetric reading using an Ilford 604 Spectrum Green filter (500–540 m μ .) against time of heating at 100° (Fig. 3) shows a rapid increase in colour up to 40 min. After this period the rate of increase is slight. The colour of the mixture, after 40 min., alters visibly to blue. A time of 30 min. was finally chosen.

Choice of filter. A study of the absorption curve in the visible region of the spectrum (Fig. 4) given by the urinary mucoprotein shows maximum absorption at 540 m μ . under the conditions described. A Spectrum Green filter (Ilford 604) was therefore adopted. These results are essentially the same as those obtained by Ayala *et al.* (1951) for the purple colour reaction given by the diphenylamine reagent

Table 2. *Specificity of diphenylamine reaction for mucoprotein*

Compound	Chromogenic power on heating with diphenylamine reagent for 30 min. at 100°
Urinary mucoprotein	100
Bovine serum albumin	2.6
Bovine serum γ -globulin	3.5
Glycogen, hyaluronic acid, glucosamine hydrochloride, L-fucose, D-glucose, D-mannose, D-galactose, nitrate	<4.0
Nitrite	Yellow turbid solution
Heparin	11.0
D-Fructose, inulin, raffinose, sucrose	100–500

Fig. 3. Rate of development of colour on heating mucoprotein A₂ with the diphenylamine reagent at 100°.

and human serum. Fig. 4 also shows the curve obtained when a fructose solution was treated similarly. Three maxima can be seen, at 500, 550 and 620 m μ . In conformity with this the resulting colour with fructose appears more blue than does the colour with urinary mucoprotein.

Method of estimation

The method of estimation was based on the second of the two methods of isolation described above since it was found that this method gave better recoveries of added mucoprotein than did the method involving the use of acetone. It was found that the variation of the pH of the mixture was critical, and that the best results were obtained by using a molar ratio of sodium benzoate to HCl of 1.25, with a resulting pH of approximately 4.5. These conditions are similar to those employed by Rimington & Rowlands (1941) for the preparation of pregnant-mare serum gonadotrophin. For pathological urines when sulphosalicylic acid is used this ratio is, however, modified to 5 to give the same pH value.

The details finally adopted were as follows:

Sulphosalicylic acid (10 ml. of 10%) was added to 40 ml. of urine in a 100 ml. conical flask. After standing for 15 min. the mixture was filtered through no. 1 Whatman filter paper. If the filtrate was not absolutely clear it was returned to the mixture in the original flask, brought rapidly to the boil and allowed to cool before refiltering. [This preliminary treatment with sulphosalicylic acid served to remove heat-coagulable protein, and to dissolve phosphate deposits if present. This procedure was not necessary in the case of clear normal urines, which were dealt with as previously described (Anderson & MacLagan, 1954).] 40 ml. of the filtrate ($\approx$ 32 ml. of the original urine) were normally taken for estimation. 5 ml. of 2M sodium benzoate were added, followed after mixing by 1 ml. of 2N-HCl. After mixing and standing for 15 min. the mixture was filtered through no. 1 Whatman filter paper on a Hirsch funnel and the precipitate of benzoic acid drained at the pump. The precipitate was then transferred quantitatively to a 10 ml. centrifuge tube

Fig. 4. Absorption spectra of diphenylamine colour with urinary mucoprotein (0.75 mg.) and fructose (0.44 mg.) in final volume of 6 ml. Unicam spectrophotometer SP. 500, $l=1$ cm.

and 8 ml. of acetone were added. After stirring to dissolve the benzoic acid, the mixture was centrifuged and the supernatant fluid rejected.

To the residue was added 2 ml. of water previously adjusted to approximately pH 9-9.5 with 2N-NaOH. This rough adjustment of pH served to accelerate solution of the mucoprotein. After stirring for 10 min. to dissolve the mucoprotein, 8 ml. of acetone were added followed by 1 drop of a saturated solution of NaCl. The mixture was stirred thoroughly and after complete flocculation (about 10 min.) was centrifuged and the supernatant fluid rejected. The residue was treated with two successive 4 ml. portions of water previously adjusted to pH 9-9.5 as described above, the mixture being stirred and then centrifuged after each addition. The two supernatant fluids obtained (total volume 8 ml.) were combined and mixed.

A convenient volume (for normal urines 3 ml.) was pipetted into a special reaction tube described above. When a smaller sample was used (which was found necessary in the case of pathological urines with a high mucoprotein content), water was added to make the volume up to 3 ml. The diphenylamine reagent (3 ml.) was then added. The tube was stoppered with the glass-stopper in the closed position, the contents mixed, and after turning the glass-stopper to the open position, heated at 100° in a boiling-water bath for exactly 30 min. It was then cooled under running water and the colour read in a photoelectric colorimeter (EEL absorptiometer, Evans Electroselenium Ltd., Harlow, Essex) using a Spectrum Green filter (520 m μ).

A blank of 40 ml. of water was treated similarly. For urines with a mucoprotein concentration exceeding 20 mg./100 ml. the estimation was carried out with a smaller volume of urine (10-20 ml.), diluted to 40 ml. with water. The reason for this is given below.

Calibration curve. Fig. 5 shows the calibration curve obtained when increasing quantities of the urinary mucoprotein in 40 ml. of water were subjected to the standard procedure. A linear relationship exists between extinction and amount of mucoprotein up to 3 mg./3 ml. of extract. Above this amount the curve deviates from a straight line. This effect is interpreted as being due to the inability of the benzoic acid to adsorb all the mucoprotein present at higher concentrations.

Fig. 5. Calibration curve for mucoprotein determination with diphenylamine reagent after benzoic acid adsorption.

Table 3. Recovery of mucoprotein added to normal urine

Initial conc. (mg./40 ml. urine)	Amount of mucoprotein added (mg.)	Final conc. (mg./40 ml. urine)	Recovery (%)
3.84	0.5	4.29	90
3.84	1.0	4.85	101
3.84	1.5	5.31	98
3.84	2.0	5.76	96
3.84	3.0	6.88	101
3.84	5.0	8.88	101

Calculation. From the calibration curve the amount of mucoprotein corresponding to the corrected colorimetric reading is read off. This is multiplied by the appropriate dilution factor to give the concentration in mg./100 ml. of urine.

The dilution factor is normally $8.33 = \frac{8}{3} \times \frac{40}{32} \times 2.5$.

Recovery of mucoprotein added to urine. Table 3 shows the recoveries obtained on adding varying amounts of mucoprotein to 40 ml. of normal urine. Recoveries of amounts between 1 and 5 mg. were approximately quantitative.

Effect of proteinuria. In urines containing heat-coagulable protein, the recovery of added mucoprotein is low, presumably owing to adsorption. Thus it was found that the addition to 40 ml. of normal urine of bovine serum albumin (100 mg.) and of bovine serum γ -globulin (100 mg.) resulted in a 31% reduction in the apparent mucoprotein content.

Method of preserving urine. Experiments were undertaken to determine the best urine preservative. Since the method of estimation is pH dependent, acid could not be used. The addition of copper sulphate was found to interfere with the colour reaction, while the use of toluene was avoided since it often forms troublesome emulsions. Thymol was found not to interfere with the colour reaction and the effect of this preservative was studied more fully. It was found that normal urine in the presence of thymol (1 g. of powdered thymol/l.) was stable for 5 days at room temperature although, surprisingly, appreciable apparent loss of mucoprotein occurred at 4°. Artificially infected urine (*Esch. coli*) gave similar results. After 5 days a slow rise in mucoprotein was observed, presumably bacterial in origin.

All urines examined were therefore preserved with thymol (1 g./24 hr. urine specimen) and stored at room temperature. The estimation was performed within 24 hr. of collection.

Variations of excretion of mucoproteins

Diurnal variation. A study of the rate of excretion at various times of day is shown in Fig. 6. Four individuals were investigated and the thick line represents the average values. Considerable variations existed both for a particular individual and between individuals. No significant rhythm could be detected.

Day-to-day variations. Fig. 7 shows the variation in the daily excretion of urinary mucoprotein measured at irregular intervals over a period of 5 months for one individual. The values varied between 111 and 190 mg./24 hr., the two peaks A and B corresponding to periods in which the subject

had a cold, symptoms of both colds first appearing on days 0 and 86, and lasting in each case for about 14 days. If the 'cold' periods are excluded the values fell within the narrower range of 111–151 mg./24 hr. When the ratio mg. mucoprotein/24 hr./S.G. is plotted, where S.G. represents the second and third decimal place of the specific

gravity reading, a similar rise in the case of the second 'cold' period was observed.

Daily excretion of urinary mucoprotein by normal subjects. Ten normal men and ten normal women were studied. Determinations were made on thymol-preserved 24 hr. urine collections which were proved protein-free by the sulphosalicylic acid test and sugar-free by Benedict's qualitative reagent. The results are presented statistically in Table 4. There is a significant difference in the amount excreted by the two sexes, the mean figure for males exceeding that for females by 40 mg./24 hr.

DISCUSSION

Confusion has existed in the past as to the exact definition of those proteins which are conjugated with hexosamine-containing carbohydrates. According to the classification of Meyer (1945), which has been extended by Stacey (1946), mucoids or mucoproteins are those substances which contain a mucopolysaccharide in firm chemical union with a peptide, where the hexosamine content is more than 4%. (A mucopolysaccharide is defined as a polysaccharide which contains hexosamine as one component but no amino acids.) Mucoids and mucoproteins are constituents of mucin, the term 'mucin' being best used in a physiological sense to denote a viscous secretion. The group of proteins which contains less than 4% hexosamine and about 1 or 2% only of firmly bound carbohydrate are termed glycoproteins and include many proteins listed as albumins and globulins. This distinction, based on hexosamine content, is arbitrary. It is convenient because those substances with more than 4% hexosamine tend to remain soluble in water after precipitation with ethanol, while those compounds with less than 4% hexosamine generally become insoluble after ethanol precipitation.

More recently a further distinction between mucoids and mucoproteins was made by Morgan (1953). Those substances which contained 50–70% carbohydrate as the major component he called mucoids, mucoproteins being those substances with considerably less carbohydrate. Examples of the former group are those substances responsible for the blood-group characters (Morgan, 1953), examples of the latter group being the carbohydrate containing proteins of serum (Winzler, Devor, Meyl & Smyth, 1948), of egg-white (Stacey & Woolley, 1942) and gonadotrophins (Gurin, 1942). This distinction between mucoids and mucoproteins is also arbitrary and there is probably no sharp distinction between them.

Urinary mucoproteins are not coagulated by heat and are not precipitated by most of the usual protein precipitants. It is probably for these reasons that their existence in normal urine has remained undetected for so long. On the other

Fig. 6. Diurnal variation in mucoprotein excretion in four subjects.

Fig. 7. Variation in urinary mucoprotein excretion by one individual; influence of respiratory infection.

Table 4. *Urinary mucoprotein excretion in normal subjects*

Sex	No. of determinations	Mg./24 hr.	
		Mean	S.D.
M	10	146	23.7
F	10	106	20.1

Significance of difference 40 ± 9.8 , $P < 0.001$.

hand, glycoproteins (albumin and globulin) have been known to occur as a trace constituent in normal urine for some time (Möerner, 1895; Addis, 1927; Wang & Wu, 1937; Gunton & Burton, 1947; Rigas & Heller, 1951; Tarnoky, 1951). The concentrations found have varied between 1 and 11 mg./100 ml. of urine. It now appears that in addition to this trace of glycoprotein in normal urine there is also present equal or slightly larger amounts of mucoprotein.

From the analytical data that has been presented mucoproteins A_1 and A_2 are essentially identical. The qualitative composition is similar to that of the purified mucoprotein obtained from normal urine by Tamm & Horsfall (1950, 1952), at least so far as the carbohydrate residues are concerned. In both cases D-galactose, D-mannose and L-fucose have been shown to be present. No reports have previously appeared on the amino acid content of the urinary mucoproteins. Our preparation appears to contain two or three more amino acids than those reported in salivary gland mucoprotein (McCrea, 1953) and in the blood group mucoids (Annison & Morgan, 1952).

Very little is known of the origin and function of the urinary mucoproteins. Mucoproteins are known to occur in serum (Winzler *et al.* 1948) and some, at least, of the urinary mucoproteins may be derived from this source. They may also be derived from the epithelium of the urinary tract and its accessory glands (Ross, Moore & Miller, 1942) and in certain cases from bacterial synthesis. These possibilities are being further investigated.

The estimation of the urinary mucoproteins by means of the diphenylamine reaction appears to be specific under the conditions laid down. The only other substances tested which gave significant positive reactions were fructose and fructose-containing compounds. The addition of fructose to urine, however, does not interfere with the reaction since it is quantitatively removed in the process of estimation. In any case, these compounds are not normal constituents of human urine.

No significant diurnal rhythm in urinary mucoprotein excretion could be detected, although wide individual fluctuations were noted at different times of day. The estimation of urinary mucoprotein excretion by one individual over a period of several months showed considerable variations in 24-hourly excretion. A part of these variations appeared to coincide with the onset of respiratory infections, the excretion falling to within lower limits during the intervening period.

Males excrete significantly more mucoprotein than females. This result was rather unexpected and possible causes are being explored. One explanation may be the presence in male urine of a certain amount of prostatic or seminal secretion. The presence of a non-heat-coagulable protein in semen

was reported as far back as 1888 by Posner, who termed it a proteose. Slowtsoff (1902) and Goldblatt (1935) also reported traces of mucin, while Wislocki (1950) found histochemical evidence of a mucoprotein in seminal plasma, but not in the spermatozoa. Ross *et al.* (1942) isolated from seminal plasma a substance which contained 10% N and 27% reducing substance. Using the method of benzoic acid adsorption we have isolated from seminal fluid a non-dialysable, non-heat-coagulable protein with the qualitative properties of a mucoprotein and which appears to migrate electrophoretically at pH 8.6 with a mobility intermediate between that of the serum β - and α_2 -globulins. From our preliminary experiments, however, it seems unlikely that this could account quantitatively for the comparatively large sex difference in excretion which has been observed. Thus a pooled specimen of semen contained approximately 900 mg. of mucoprotein/100 ml., and the chromogenic power of this material with diphenylamine was about one-third that of the urinary mucoprotein. Hence, to account for the sex difference, some 13 ml. of seminal fluid/l. of male urine would apparently be required.

The significance of urinary mucoprotein has been little studied in the past owing to the lack of a simple method of estimation, and it is hoped that the use of the method described here will facilitate the investigation of this problem.

SUMMARY

1. A method of isolating mucoproteins from normal male urine by the use of adsorption on benzoic acid has been investigated.
2. Two similar mucoprotein preparations (A_1 and A_2) were obtained in a yield of approximately 33 mg./l. of urine. Their properties have been investigated and analytical results are presented.
3. A colorimetric method for the estimation of urinary mucoproteins in normal and pathological urines is described.
4. No significant diurnal excretory rhythm was observed in four normal subjects studied. Observations over a period of 5 months on one normal subject are recorded.
5. Ten normal male subjects excreted 146 ± 7.5 mg. of mucoprotein in 24 hr., while ten normal females excreted 106 ± 6.4 mg. The sex difference was statistically significant (40 ± 9.8) and a possible cause for this is discussed.

This work was aided by grants from the British Empire Cancer Campaign and from the Governors' Discretionary Fund of Westminster Hospital, which we gratefully acknowledge. We are also indebted to Miss Pamela Hasted and Miss Diana Lawson for skilled technical assistance.

Note added in Proof. While this paper was in the press, we noted the work of Werner & Odin (1952) on the reaction of diphenylamine with crystalline sialic acid. The absorption curve which they report for the coloured complex produced is very similar to that shown above in Fig. 4, and it appears likely that our method of estimating urinary mucoprotein depends mainly upon its sialic acid content.

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Studies on the Heterogeneity of Crystallized β -Lactoglobulin

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(Received 1 September 1954)

In spite of evidence to the contrary, it seems to have been assumed that β -lactoglobulin of cow's milk is sufficiently homogeneous to make its amino acid analysis (Brand & Kassell, 1942; Brand, Saidel, Goldwater, Kassell & Ryan, 1945; Stein & Moore, 1949) and its physico-chemical properties (Cohn & Edsall, 1943) worth measuring. This view may have been encouraged by the ease with which clean crystalline preparations can be made.

Evidence for its heterogeneity has come primarily from electrophoretic studies. Pedersen (1936) first reported that it migrated as a single component over a wide range of pH; these measurements were made at 20° and at low concentration of protein. All subsequent work has been at 0.5–2° and at higher concentration of protein (Li, 1946, 1951; Polis, Schmukler, Custer & McMeekin, 1950;

McMeekin, Polis, DellaMonica & Custer, 1948; Alberty, Anderson & Williams, 1948; Bain & Deutsch, 1948; Deutsch, 1950; Jacobsen, 1949; Smithies, 1954). Direct comparison of the results of these workers is difficult because of the variety of conditions of pH and ionic strength used; however, all agree that β -lactoglobulin migrates as two or sometimes three components and that two main components can be distinguished in acetate and acetate-chloride buffers of pH 4.2–4.8. Polis *et al.* (1950) showed that the ratio of acetate to chloride appears to affect both the mobilities and the proportions of the two components at pH 4.8. Some authors have also commented on a marked difference between the ascending and descending boundaries, especially at pH 4.8 and lower. Lundgren & Ward (1949) have suggested that the heterogeneity might be due to contamination with other whey proteins, but Polis *et al.* (1950) rejected

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