purification experiments were severely hampered by the lability of the enzyme.

2. Peptide-bond formation specifically required adenosine triphosphate, and magnesium ions could be replaced to a considerable extent by manganous ions. Carnosine synthesis was not readily reversible. Pyrophosphate was strongly inhibitory, and orthophosphate less so. Although the enzyme preparation promoted [^{32}P]pyrophosphate incorporation into adenosine triphosphate, a dependency upon β-alanine could not be clearly demonstrated. In fact, this process was suppressed by a high β-alanine concentration. Fluoride ions inhibited both carnosine synthesis and pyrophosphate–adenosine triphosphate exchange.

3. Attempts to detect an activated intermediate stage in carnosine synthesis were not successful.

4. β-Alanyl transfer from carnosine to 1-methylhistidine to yield anserine proceeded very slowly, as compared with direct dipeptide synthesis by the enzyme.

This research was supported by a grant from the Muscular Dystrophy Associations of America.

REFERENCES

Davis, J. W. & Novelli, G. D. (1958). *Arch. Biochem. Biophys.* **75**, 299.
Drury, H. F. (1948). *Arch. Biochem.* **19**, 455.
Johnston, R. B. & Bloch, K. (1951). *J. biol. Chem.* **188**, 211.
Kalyankar, G. D. & Meister, A. (1959). *J. biol. Chem.* **234**, 3210.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
Novelli, G. D. (1959). *Biochim. biophys. Acta*, **33**, 261.
Snoke, J. E. (1955). *J. biol. Chem.* **213**, 813.
Snoke, J. E. & Bloch, K. (1954). In *Glutathione*, p. 129. Ed. by Colowick, S. P. *et al.* New York: Academic Press Inc.
Tiselius, A., Hjertin, S. & Levin, O. (1956). *Arch. Biochem. Biophys.* **65**, 132.
Webster, G. C. & Varner, J. E. (1955). *Arch. Biochem. Biophys.* **55**, 95.
Winnick, R. E. & Winnick, T. (1958). *Bull. Soc. Chim. biol., Paris*, **40**, 1727.
Winnick, R. E. & Winnick, T. (1959*a*). *Biochim. biophys. Acta*, **31**, 47.
Winnick, R. E. & Winnick, T. (1960). *Biochim. biophys. Acta*, **37**, 214.
Winnick, T. & Winnick, R. E. (1959*b*). *Nature, Lond.*, **183**, 1466.

Biochem. J. (1960) 77, 581

The Fractionation of Urine Colloids on Anion-Exchange Cellulose

By A. J. ANDERSON,* M. H. LEPPER and R. J. WINZLER
Departments of Biological Chemistry and Preventive Medicine, University of Illinois College of Medicine, Chicago, Illinois, U.S.A.

(*Received* 30 *March* 1960)

Urine contains a complex mixture of high-molecular-weight material, some components of which may be derived from the serum through a process of glomerular filtration or secretion and some of which may be derived from the urinary tract itself. Information about the nature of the urine colloids, however, has lagged considerably behind the knowledge of those in serum. Three main classes of high-molecular-weight compounds in urine have been recognized: proteins, glycoproteins and mucopolysaccharides. The presence of protein in urine was recognized as early as 1895 by Möerner and urine proteins have since been studied extensively by many workers, particularly Rigas & Heller (1951) and McGarry, Schon & Rose (1955). The variation in the amount of heat-coagulable protein in urine with several diseases, particularly the nephrotic syndrome, is well known (Heremans, 1958).

The term 'glycoprotein' is used to indicate components containing appreciable amounts of hexosamine, which can be liberated only after vigorous hydrolysis. Glycoproteins generally also contain hexose and sialic acid, with little or no uronic acids. Urinary fractions presumed to contain glycoproteins have been studied by Boyce, King, Little & Artom (1958), Boyce & King (1959) and King, Boyce, Little & Artom (1958). One normal urinary glycoprotein, first described by Tamm & Horsfall (1950), has been extensively characterized with respect to its composition and physical properties (Tamm & Horsfall, 1952; Tamm, Bugher & Horsfall, 1955; Porter & Tamm, 1955). Anderson (1954) has reported the isolation of two heterogeneous glycoprotein fractions from normal urine. Application of a method for estimating one of these fractions (Anderson & Maclagan, 1955)

* Present address: Department of Pathology, Royal National Orthopaedic Hospital, Stanmore, Middlesex, England.

showed considerable variation from normal in a number of diseases (Lockey, Anderson & Maclagan, 1956). This fraction was found to possess a wide range of biological properties, including antitryptic, gonadotrophic, amylase and vitamin B_{12}-binding activities, and to be a potent inhibitor of viral haemagglutination (Anderson, Lockey & Maclagan, 1955). In addition, the presence of urokinase and uropepsinogen activities has been observed (Kickhöfen, Struwe, Bramesfeld & Westphal, 1958) in this fraction. The fact that similar biological properties have been reported for serum glycoprotein fractions suggests that some of the urine glycoproteins may be derived from serum.

Mucopolysaccharides, such as chondroitin sulphate, also occur in urine. These may occur free of protein or may exist as mucopolysaccharide–protein complexes similar to those observed in cartilage by Shatton & Schubert (1954). There have been several reports of the presence of these colloids in normal urine (Rich & DiFerrante, 1956; DiFerrante & Rich, 1956; Jacques, Bapke & Levy, 1953; Kerby, 1954), and of their elevated levels in several diseases (Craddock & Kerby, 1955).

Several reports are available on the nature and amount of the total colloids both in normal urine and various pathological conditions (Hammerman & Hatch, 1955; King *et al.* 1958; Maclagan & Anderson, 1958*a*; Björnesjö, Werner & Odin, 1959). Electrophoretic and chemical studies have been carried out on several heterogeneous urine-colloid fractions obtained after salt-fractionation procedures (Boyce, Garvey & Norfleet, 1954; Boyce *et al.* 1958; Boyce & Swanson, 1955; Boyce & King, 1959; King *et al.* 1958; King, Little, Boyce & Artom, 1959). It is likely that a more complete fractionation and characterization of the high-molecular-weight components in urine will be followed by valuable information about their source, physiological significance and clinical importance.

This paper describes the fractionation of non-ultrafiltrable, non-dialysable urine components on cellulose ion-exchange columns and a study of some of the chemical and physical properties of some of the fractions so separated. An investigation of the ability of these fractions to bind calcium is also included, together with the calcium-binding ability of the colloids in complete 24 hr. specimens of both normal and pathological urines.

METHODS

The methods used in these studies included the following. Urine glycoproteins: Anderson & Maclagan (1955); sialic acid: Werner & Odin (1952); hexose: Winzler (1955); hexosamine: Rondle & Morgan (1955); fucose: Dische & Shettles (1948); nitrogen: micro-Kjeldahl; calcium: Ferro & Hamm (1957); hexuronic acid: Dische (1947); protein: Lowry, Rosebrough, Farr & Randall (1951); qualitative and quantitative precipitin reactions: Kabat & Meyer (1948); immunoelectrophoresis: Williams & Grabar (1955); paper electrophoresis: Durram (1950), Flynn & De Mayo (1951); chloride: by a method involving the polaragraphic estimation of residual silver ions when a chloride-containing solution is added to silver nitrate in 0·1 N-nitric acid.

Inhibition of virus haemagglutination. Inhibitor activity was determined with Lee B influenza virus harvested from allantoic fluid and converted into the indicator phase by heating at 56° for 30 min. Serial twofold dilutions of the virus preparation were made in 5 mM-phosphate buffer, pH 7·0, and to 0·5 ml. of each dilution was added 0·25 ml. of 0·5% suspension of washed, type-O human erythrocytes. After mixing, the tubes were incubated at 4° overnight. The last dilution of the virus to show agglutination was taken as 1 haemagglutination (HA) unit. Serial twofold dilutions were made from portions of the fractions taken from the DEAE-cellulose column, and to 0·25 ml. samples of these dilutions were added 0·25 ml. of virus suspension containing 8 haemagglutinating units of the indicator virus. After incubation for 4 hr. at 4°, 0·5 ml. of a 0·5% suspension of erythrocytes was added, and the tubes were read after incubation at 4° overnight. The last dilution to inhibit haemagglutination was defined as 1 inhibiting (HAI) unit. The specific activity of each fraction was expressed as HAI units/μg.

Determination of calcium-binding. Calcium-binding was determined by an equilibrium-dialysis technique in which a known amount of the colloid under investigation (about 10 mg.) was dissolved in 11 ml. of veronal buffer (0·01 M, pH 9·0) and dialysed against 20 ml. of the veronal buffer containing 5 mM-calcium chloride. After equilibration by slow shaking overnight at 4°, the concentrations of calcium and chloride (μequiv./ml.) both inside and outside the dialysis sac were determined. The amount of bound calcium inside the sac was then calculated, assuming the existence of the simple Donnan equilibrium, the amount of calcium bound by the colloid fraction being expressed as μequiv. of calcium/mg. of colloid. The same method was used for estimating the calcium-binding by urine specimens, 100 ml. of the 24 hr. urine output first being dialysed, freeze-dried and then taken up in 11 ml. of the veronal buffer. In this case results were expressed as the amount of calcium (μequiv.) bound by the colloids in the complete 24 hr. urine specimen.

Ultrafiltration of the urine colloids. Urine, preserved with thymol, was stored for 24 hr. at 4° and insoluble material removed by filtration. The urine was then ultrafiltered in 4 l. batches under air pressure at 65 lb./in.^{2} at 4° through collodion membranes of diam. 15·5 cm. (Schleicher and Schüll, L.S.G. 60) capable of retaining spherical molecules of molecular weights of 10 000 and over. The used membranes were immediately stored at −20°.

Separation of the urine colloids from the membranes. In a typical experiment the colloids obtained from 16·6 l. of pooled normal male urine were extracted from the membranes by soaking them three times for 24 hr. at 4° in 100 ml. of the starting buffer (SB) described in Table 2. The pooled washings were dialysed at 4° against running distilled water for 72 hr. and were freeze-dried, yielding 960 mg. of fraction A (58 mg./l.). The membranes were

then shredded and dissolved in 300 ml. of an ethanol–ether mixture (1:1) at 4°; the solution was mixed with an equal volume of 0·1 N-NaOH, filtered and the ether removed by aeration. The solution was dialysed at 4° for 72 hr. against running distilled water, and freeze-dried to yield 2340 mg. of fraction B (141 mg./l.). An additional 12 mg./l. did not dissolve in 0·1 N-NaOH after dissolution of the membranes. The protein and carbohydrate composition of fractions A and B is shown in Table 1.

The composition of these fractions suggests the presence of considerable amounts of carbohydrate-rich components, as previously found by Hammermann & Hatch (1955), Boyce *et al.* (1954), Boyce & King (1959), King *et al.* (1958, 1959) and others. The differences in the composition of fractions A and B may in part be due to molecular size, with relatively more of fraction B being in the interstices of the collodion membrane, and in part to the solubility properties, fraction B being dissolved in 0·1 N-NaOH and fraction A in the starting buffer at pH 9·0. For subsequent fractionation the non-dialysable residues A and B were combined.

Fractionation of the urine colloids. The procedure developed by Sober, Cutter, Wyckoff & Peterson (1956) was employed, the anion-exchange cellulose adsorbent diethylaminoethylcellulose (DEAE-cellulose) being used.

DEAE-cellulose (80–140 mesh, 0·71 m-equiv. of ionizing groups/g., obtained from Brown Co., Berlin, New Hampshire, U.S.A.) was washed successively with N-NaOH, N-HCl and N-NaOH, filtration under suction being used each time. After washing to neutrality with water, it was then washed with the starting buffer described in Table 2. The exchanger was then poured as a slurry into a glass tube (length 42 cm., diam. 2·8 cm.), the lower end of which contained a layer of glass wool supported on a coarse metal grid. After the cellulose had settled by gravity the column was further compacted by application of air pressure at 5 lb./in.^{2} By this means, 40–50 g. of cellulose exchanger formed a packed column 35–40 cm. high. Several litres of the starting buffer were then passed through the column. The flow rates under gravity and under air pressure of 5 lb./in.^{2} were 0·74 and 3·3 ml./min. respectively.

Urine colloids (1–3 g.) were suspended in about 25 ml. of starting buffer, and the mixture was stirred continuously for 18 hr. at 4°. Insoluble material was centrifuged off and resuspended in fresh buffer, stirring as before, this process being repeated three times. The combined supernatant fluids contained 70% of the total non-dialysable residue. This solution was then applied to the top of the column and allowed to enter under gravity, and was washed in with about 20 ml. of starting buffer.

A pH and ionic-strength gradient was then applied to the column, the Varigrad mixing device developed by Peterson & Sober (1958) being used. This consisted of nine cylinders (designated 1–9) each 7·5 cm. in diam. and 13 cm. high. These were connected at their bases in series, the outlet from cylinder 1 leading to the top of the cellulose column via a pumping device through which the eluting buffers were supplied. Cylinders 1–9 contained buffers of decreasing pH and increasing ionic strength.

A 2-amino-2-hydroxymethylpropane-1:3-diol (tris)–citrate buffer system was devised, for use with the Varigrad apparatus, which would deliver buffer to the cellulose column at a continuous pH descent from 9 to 3, accompanied by an increasing ionic strength. The compositions of the starting buffer, limit buffer and the Varigrad mixtures are shown in Table 2. After about 3 l. had passed through the column, three further eluting solutions were used (buffers 2–4), the final effluent emerging at a pH of 1·5. [Buffer 2 (pH 2·25): tris, 30·54 g.; citric acid, 205·0 g.; sodium chloride, 5·85 g.; water, 750 ml. Buffer 3 (pH 1·46): citric acid, 210·14 g.; water, 1000 ml. Buffer 4 (pH 1·41): citric acid, 210·14 g.; sodium chloride, 58·5 g.; water, 1000 ml.] At pH values below 2·0 considerable retraction of the cellulose occurred, necessitating a gradually increasing air pressure up to a maximum of 80 lb./in.^{2} in order to maintain a conveniently rapid rate of flow. The effluent was collected in individual volumes of 10 ml., with a fraction collector, the total volume being about 4 l. The complete chromatographic separation was accomplished in 24–30 hr.

The fractions from the column were examined in a Beckman spectrophotometer at 280 mμ. The fractions comprising the various peaks were then combined, filtered, dialysed against running distilled water at 4° for 3 days and were freeze-dried.

RESULTS

Normal urine

Fig. 1 shows a chromatogram of the urine colloids from 13·3 l. of pooled normal male urine (1860 mg.), isolated as described above. A number of poorly resolved peaks are evident.

Table 1. *Composition of some components in the non-dialysable fractions A and B*

	Non-dialysable residue A (%)	Non-dialysable residue B (%)
Sialic acid	6·2	1·0
Hexose	8·4	39·0
Hexosamine	15·8	2·0
Hexuronic acid	1·4	4·4
Protein	37·0	14·0

Table 2. *Buffer system used for the separation of urine colloids on DEAE-cellulose column*

Buffer 1: starting buffer (SB) contained (in 1 l. of water): tris, 24·429 g.; citric acid, 1·0507 g. Limit buffer (LB) contained (in 5 l. of water): tris, 122·14 g.; citric acid, 315·21 g.; sodium chloride, 5·85 g. The table shows the compositions of buffers used for the Varigrad apparatus. Buffers 2–4 are described in the text.

Cylinder no.	SB (ml.)	LB (ml.)	pH
1	400·0	0	9·00
2	397·5	2·5	7·89
3	397·5	2·5	7·89
4	393·0	7·0	5·46
5	393·0	7·0	5·46
6	393·0	7·0	5·46
7	385·0	15·0	4·30
8	150·0	250·0	3·01
9	0	400·0	2·79

The composition of the fractions, pooled as indicated, is given in Table 3. Only about 38% of the colloids placed on the column could be recovered in the effluents, indicating that a considerable amount of irreversible absorption on to the DEAE-cellulose had occurred. The protein percentages obtained by the method of Lowry *et al.* (1951) correlated fairly well with the amount of light-absorption at 280 mμ (Fig. 1). Both these procedures depend primarily on the presence of aromatic amino acids. Protein percentages calculated from the total nitrogen values, however, were consistently higher, particularly for fractions 10, 14 and 15, indicating that these fractions were probably deficient in aromatic amino acids. It would therefore appear that protein percentages calculated from the total nitrogen figures are a more reliable index of true protein content. Each of the fractions showed the presence of considerable amounts of carbohydrate. Fractions 1, 2 and 9 were characterized by being especially rich in fucose, fractions 9, and 12–15 in hexuronic acid and 6, 9–10 in sialic acid.

Although the hexose:hexosamine:sialic acid ratios of fractions 1–8 varied somewhat, in general they approximated more closely to those that have been reported for the serum proteins (Winzler, 1955; Sonnet, 1956; Laurent, 1958) than did the corresponding ratios for fractions 9–15. The higher proportions of hexuronic acid in the later fractions indicated an elution of acid mucopolysaccharides. Non-dialysable calcium was associated with all fractions but especially in the later fractions, with the exception of fraction 12. Fractions 9, 10 and 11, showing little absorption at 280 mμ, contained 62% of the calcium eluted from the column.

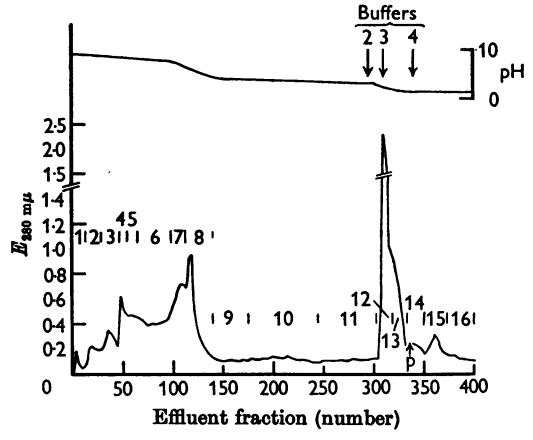

Fig. 1. Chromatogram obtained from the fractionation of the colloids from normal male urine on DEAE-cellulose. 'p' indicates a fraction that precipitated spontaneously from solution immediately after emerging from the column.

Table 3. *Percentage composition of fractions from pooled normal male urine obtained by elution from DEAE-cellulose column*

Percentage concentration of proteins (column 11) was obtained in two ways: (*a*) calculated by multiplying the total nitrogen, after subtracting hexosamine N (7·8%) and sialic acid N (4·54%), by 6·25; (*b*) by the method of Lowry *et al.* (1951).

Fraction no.	Yield (mg.)	Concn. in effluent (mg./ml.)	Hexose (%)	Hexosamine (%)	Sialic acid (%)	Fucose (%)	Hexuronic acid (%)	Calcium (%)	Total nitrogen (%)	Proteins (%) (*a*)	Proteins (%) (*b*)	Calcium-binding (μequiv. of Ca/mg. of colloid)	Haemagglutination inhibition (HAI units/mg.)
1	41·8	0·53	9·6	6·33	3·66	2·73	1·31	0·34	—	—	—	0	0
2	22·0	0·14											0
3	23·5	0·13	10·7	6·7	5·36	1·42	0·90	0·62	10·90	63·6	54·0	0·155	0
4	36·4	0·33											0·024
5	53·8	0·26	10·0	7·2	5·67	1·02	1·53	0·72	10·20	58·8	43·6	0·016	0·25
6	53·3	0·29	13·3	9·3	8·53	1·73	0·80	0·86	—	—	58·7	0	1·8
7	68·5	0·60	6·8	5·0	3·76	0·79	0·84	0·72	13·0	78·0	52·8	0·221	0·43
8	75·2	0·29	8·4	5·3	5·28	0·93	1·14	0·92	10·9	64·3	48·5	0·385	0·44
9	28·4	0·08	12·5	9·1	8·72	3·60	2·26	2·58	8·66	47·7	31·6	0·189	0·38
10	120·8	0·19	7·5	7·4	8·68	0·77	1·35	4·07	9·93	56·4	20·2	0·637	0·68
11	67·8	0·12	4·8	3·1	3·22	0·47	1·23	3·31	—	—	18·1	0·263	0·067
12	44·4	0·34	16·8	5·9	5·56	1·34	3·06	0·46	10·54	61·7	41·2	0·249	3·30
13	32·3	0·14	11·4	3·4	2·30	1·15	2·60	2·72	9·44	56·7	32·4	0·737	0
14	21·8	0·14											0
15	20·0	0·12	11·4	4·6	1·68	1·48	5·06	2·88	7·54	44·5	18·2	0·880	0·27
Total:	710·0												

All of the fractions obtained were obviously extremely heterogeneous. This is evident in Fig. 2, which shows the results from paper electrophoresis of fractions 1–8 at pH 8·6 and 4·6.

At pH 8·6 these fractions showed a gradual increase in electrophoretic mobility, predictable from their behaviour on the ion-exchanger, protein eluting first having the lowest negative charge. Fractions 1–8 gave an intense stain with bromophenol blue, whereas fractions 9–15 stained feebly. Fractions 9–12 had mobilities mainly of α- and β-globulins whereas fractions 13 and 15 were immobile.

At pH 4·6 increasing amounts of negatively charged material staining with bromophenol blue were present in fractions 1–8. Of the feebly staining fractions 9–15, fraction 12 had negatively charged material which migrated considerably further than human-serum orosomucoid, indicating the presence in urine of colloids which so far have not been reported present in serum.

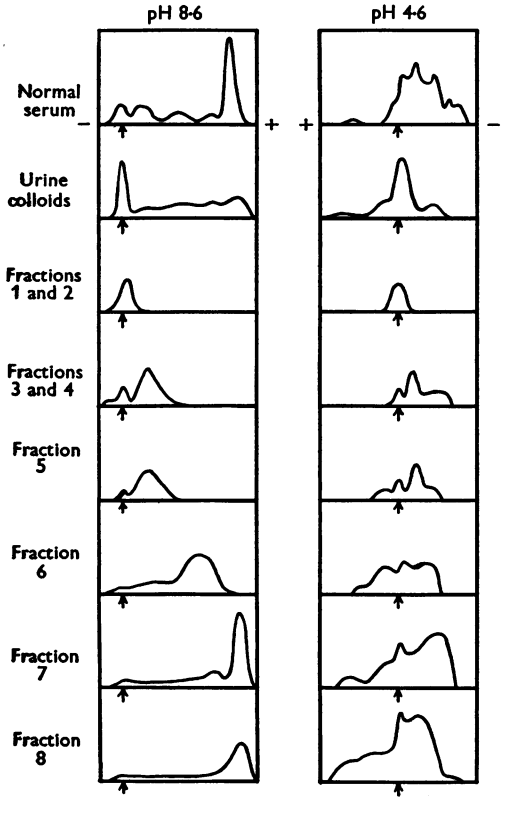

Fig. 2. Electrophoresis patterns of the fractions obtained from the separation of normal urine colloids on DEAE-cellulose. Paper electrophoresis was carried out at pH 8·6 and pH 4·6.

Some biological activities of the urine-colloid fractions

Inhibition of viral haemagglutination. Human urine is known to be a rich source of an inhibitor of influenza viral haemagglutination. Fractions high in inhibitory activity have been isolated and studied by Tamm & Horsfall (1950, 1952) by Faillard (1956) and by Anderson *et al.* (1955) among others. The urine fractions from the DEAE-cellulose column were therefore examined for their abilities to inhibit viral haemagglutination. The dialysed effluents from the column before freeze-drying were used, the colloid concentrations present being listed in Table 3 (column 3).

The last column of Table 3 shows the specific activities of the various fractions. The distribution clearly indicates the presence of several areas of activity with the highest specific activities found in fractions 6 and 12. These fractions had activities comparable with that of the glycoprotein isolated by Tamm & Horsfall (1950).

Antigenic components. Antibodies prepared in chickens to the uromucoid of Boyce & Swanson (1955), and tested by the method of Goodman *et al.* (1957), were found to react strongly with fractions 4–8 and also with 12 and 15. This suggests that the uromucoid fraction is quite heterogeneous or that the processes of fractionation resulted in a separation into degraded products or both.

Fractions 6–8, particularly the last, reacted with antisera prepared in chickens to human-serum albumin and human-serum orosomucoid, which indicated that these fractions contained these serum proteins.

Immunoelectrophoretic analyses showed the presence of one or more arcs when fractions 2–8 from pathological urine II (see below) reacted with anti-human serum. Other fractions had no arcs or very weak ones. This evidence again suggests that fractions 2–8 contain some of the proteins derived from serum.

Pathological urine I

Urine from a patient who was severely paralysed with poliomyelitis and forming bilateral renal calculi was studied. There was no evidence of proteinuria by the heat and acetic acid methods. Significant bacteriuria with a mixture of *Escherichia coli*, *Proteus mirabilis* and enterococci was present.

A volume (14·4 l.) of the urine was treated as described previously. The yield of colloids soluble in the starting buffer placed on the column was 2550 mg. (177 mg./l.). These colloids were fractionated on a freshly prepared DEAE-cellulose column under the same conditions as were used for the normal urine. The chromatogram obtained is shown in Fig. 3. The distribution of components was rather similar to that shown in Fig. 1 for

normal urine, but the proportions in the different peaks were somewhat different.

The analytical results for the fractions are presented in Table 4. The ratios of hexose:hexosamine:sialic acid were considerably different from those for normal urine, primarily as a result of lower sialic acid and hexosamine contents and a higher hexose content. Fucose content was also below the values found in the normal urine.

As with the normal fractions, increased amounts of acid mucopolysaccharide were eluted from the

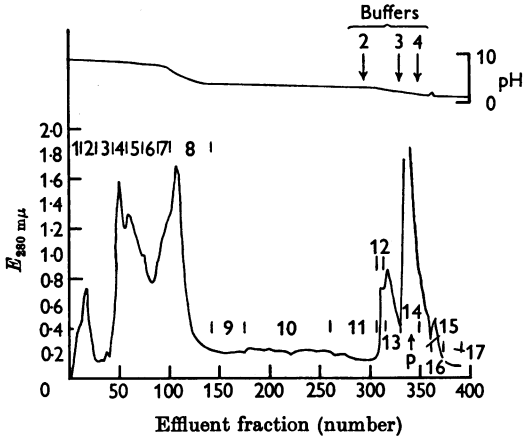

Fig. 3. Chromatogram of the colloids from a stone-forming poliomyelitis urine obtained after fractionation on DEAE-cellulose. 'p' indicates a fraction that precipitated spontaneously from solution immediately after emerging from the column.

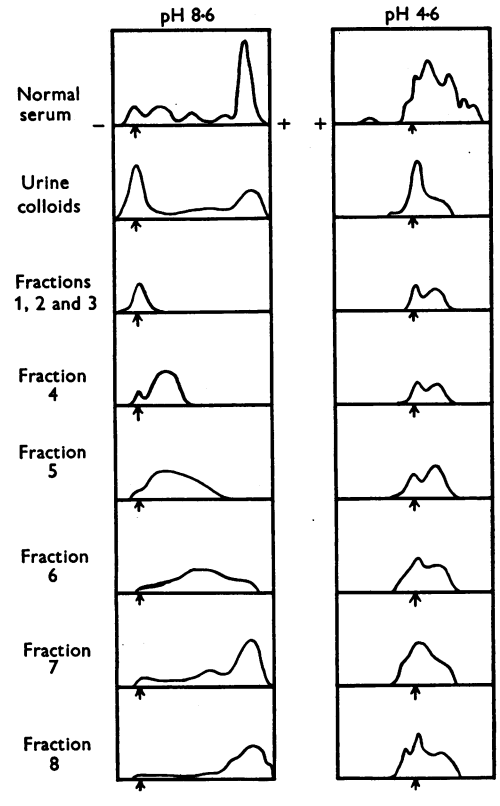

Fig. 4. Electrophoresis patterns of the fractions obtained from the separation of urine colloids from a stone-forming poliomyelitis urine. Paper electrophoresis was carried out at pH 8·6 and pH 4·6.

Table 4. *Percentage composition of fractions from poliomyelitis urine I obtained by elution from DEAE-cellulose column*

Fraction no.	Yield (mg.)	Concn. in effluent (mg./ml.)	Hexose (%)	Hexosamine (%)	Sialic acid (%)	Fucose (%)	Hexuronic acid (%)	Calcium (%)	Calcium-binding (μequiv. of Ca/mg. of colloid)
1	11·7	0·08							
2	2·8	0·01	13·8	7·55	2·34	1·86	1·02	2·42	0·252
3	7·6	0·05							
4	97·8	0·51	13·3	4·87	2·65	0·74	0·51	0·23	0
5	66·5	0·35	13·2	3·75	1·34	0·64	0·83	1·06	0·148
6	81·8	0·38	13·5	3·36	1·59	0·78	1·31	1·21	0·595
7	192·7	0·67	7·2	1·45	0·97	—	0·51	0	0·068
8	104·8	0·31	9·0	2·13	1·14	0·58	0·91	1·21	0·285
9	32·3	0·10	—	—	1·30	—	1·32	4·54	0
10	62·8	0·07	14·0	3·83	0·91	2·38	1·85	6·36	0·447
11	43·3	0·07	11·3	4·93	0·81	—	2·21	6·73	0·275
12	14·6	0·14	—	7·98	1·55	—	1·41	2·50	0·053
13	4·1	0·03							
14	104·3	0·54	13·1	3·61	2·04	1·76	2·88	0	0·094
15	36·5	0·22	23·8	3·00	1·07	—	1·67	0·91	0·366
16	31·3	0·07	12·1	3·78	0·51	1·20	4·72	9·16	0·164
17	20·7	0·06							
Total:	915·6								

column at higher ionic strengths and lower pH values; 48% of the total non-dializable calcium was located in fractions 9, 10 and 11.

Fig. 4 shows the results obtained by paper electrophoresis of these fractions at pH 8·6 and 4·6. Electrophoretic mobilities and staining properties were rather similar to those obtained for the normal urine fractions. At pH 4·6, however, less negatively charged material was present, indicating a lower acid glycoprotein content than in normal urine. This conclusion is in accord with the lower sialic acid values shown in the analytical figures in Table 4.

Reactions against antibodies to human-serum albumin were positive for fractions 6, 7 and 8 only. Reactions with anti-orosomucoid serum were positive for fractions 6 and 7, as was the case for the fractions from normal urine. In addition a strong reaction was given by fraction 4.

Pathological urine II

This urine was from a patient severely paralysed by poliomyelitis but who at the time of urine collection had never formed stones. No clinical proteinuria was present, although the urine was infected with *Aerobacter aerogenes*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and a species of *Enterococcus*. The colloids from 23·4 l. of urine were processed as described above and placed on the DEAE-cellulose column as in previous runs. The chromatogram obtained is shown in Fig. 5 and some analytical results for the various fractions are shown in Table 5. The chromatogram showed a distinctly different pattern from that seen in the normal urine or in pathological urine I, with much more material coming out in peak 1.

As in the previous two urines a high proportion of the non-dialysable calcium (32%) was associated with fractions 9, 10 and 11, which contained little ultraviolet-absorbing material.

Reactions against antisera to human-serum albumin and human-serum orosomucoid were positive for fractions 7 and 8 only. This urine contained a relatively larger amount of fraction 1 and a lower amount of fractions 7 and 8 than the previous two urines. As already mentioned, fractions 2–8 contained several antigens reacting with antibodies prepared to normal human serum.

Investigation of the urine-colloid fractions for calcium-binding ability

In an effort to determine whether the total calcium-binding capacity in patients deviated from normal and might be related to the formation of stones, the ability of various urine-colloid

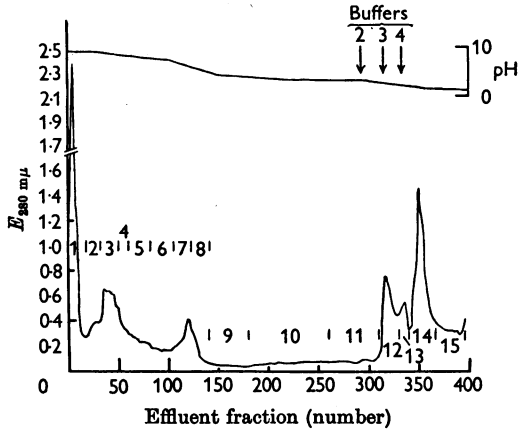

Fig. 5. Chromatogram of the colloids from a stone-free poliomyelitis urine obtained after fractionation on DEAE-cellulose.

Table 5. *Percentage composition of fractions from poliomyelitis urine II obtained by elution from DEAE-cellulose column*

Fraction no.	Yield (mg.)	Concn. in effluent (mg./ml.)	Hexuronic acid (%)	Calcium (%)
1	157·3	1·00	1·71	0
2	29·3	0·20		
3	52·5	0·24	—	0·38
4	12·8	0·11		
5	22·2	0·12	—	0·45
6	22·4	0·09	—	0·67
7	35·8	0·20	1·31	0·11
8	27·6	0·12	1·30	0·38
9	16·3	0·04	—	1·93
10	22·3	0·03	2·00	0·53
11	25·4	0·04	1·82	0·74
12	28·7	0·09	14·4	0·83
13	23·1	0·14	12·9	0·50
14	24·0	0·07	6·35	0·82
15	31·4	0·06		
Total:	531·1			

fractions to bind calcium was investigated. The colloids from 24 hr. urine specimens derived from normal subjects, and also from patients recovering from poliomyelitis (many of whom had formed stones), were used.

Table 6 illustrates some typical results obtained for several colloid fractions: $ChSO_4$ refers to the potassium salt of chondroitin sulphate isolated from nasal cartilage by the method of Einbinder & Schubert (1951); A_5 was a glycoprotein-rich fraction isolated from normal urine by the benzoic acid-adsorption method (Anderson & Maclagan, 1955); HSA refers to human-serum albumin; NUUC (normal) and (path.) refer to the non-ultrafiltrable colloids isolated by the method here described from the urine of normal subjects and from the urine of a patient with bilateral calculi.

Table 6. *Calcium-binding ability of colloids*

$ChSO_4$ is the potassium salt of chondroitin sulphate isolated from bovine nasal cartilage by the method of Einbinder & Schubert (1951). A_5 is a glycoprotein-rich fraction isolated from normal urine by the benzoic acid method of Anderson & Maclagan (1955). HSA is human-serum albumin. NUUC (normal) is the non-ultrafiltrable urine colloid isolated from normal urine by the present method. NUUC (path.) is the corresponding fraction isolated from the urine of a patient with bilateral calculi.

Colloid	Bound calcium (μequiv./mg. of colloid)
$ChSO_4$	1·14
A_5	0·26
HSA	0·20
NUUC (normal)	0·45
NUUC (path.)	0·56

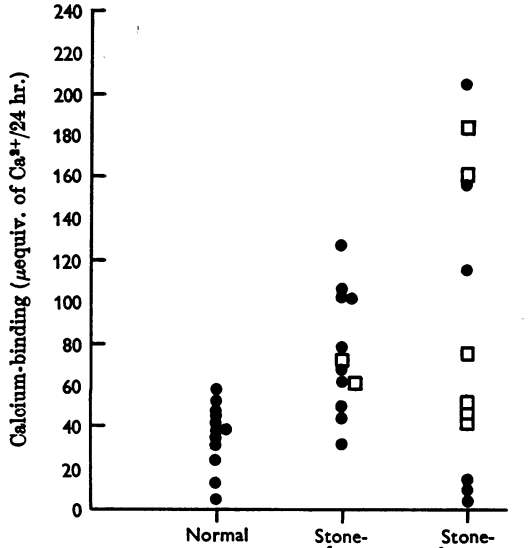

Fig. 6. Calcium-binding levels of 24 hr. urine colloids. □, Clinical proteinuria.

The calcium-binding abilities of the normal colloids obtained from the DEAE-cellulose column (Fig. 1) are shown in Table 3. Calcium-binding by the fractions from pathological urine I (Fig. 3) are contained in Table 4.

From these data no relationship is evident between the extent of calcium-binding and content of sialic acid, fucose, hexosamine or the non-dialysable calcium content of the chromatographic fractions from either urine sample. The normal colloid fractions 1–8 (Table 3) contained relatively low amounts of hexuronic acid and low calcium-binding abilities. Fractions 10–16, however, with higher contents of hexuronic acid, appeared to bind calcium somewhat more strongly. The fractions from the poliomyelitis urine (Table 4) showed less relationship between their hexuronic acid contents and their calcium-binding abilities. It is therefore clear that factors other than the content of uronic acid-containing mucopolysaccharides must be important in the binding of calcium.

The calcium-binding levels of the colloids in 24 hr. urine specimens from both normal subjects and patients recovering from poliomyelitis are shown in Fig. 6. No correlation with the duration of the disease or with the daily urine-output volume was evident.

DISCUSSION

The ultrafiltration method for isolating the colloids from normal male urine gave yields of about 200 mg./l. of urine. At pH 9·0 these colloids were soluble only to the extent of 140 mg./l. These values are in essential accord with partition noted by Boyce *et al.* (1958). About 65% of the weight of the non-ultrafiltrable fractions could be accounted for on the basis of their content of hexose, hexosamine, sialic acid, fucose, hexuronic acid and protein. The composition of these colloids, as has previously been demonstrated by Tamm & Horsfall (1952), Anderson & Maclagan (1955), Craddock & Kerby (1955), Hammerman & Hatch (1955), Baar (1956), DiFerrante & Rich (1956), Bonomo, Salteri & Cirla (1958), King *et al.* (1958, 1959), Boyce *et al.* (1958), Boyce & King (1959) and others, indicates the presence of relatively large amounts of glycoproteins and mucopolysaccharides.

The separation of the colloids by the DEAE-cellulose procedure of Sober & Peterson (1958), modified to have a steeper gradient of pH and salt concentration, has resulted in fractionation of the

urine colloids into several heterogeneous fractions of quite different composition. About 38% of the colloid added to the column was recovered in the various effluents.

The three chromatograms obtained by use of the tris–citrate buffer system that was devised (Table 2) can be divided into three distinct parts. Some components of the first eight fractions emerging appeared to contain aromatic amino acids, and on immunochemical evidence were in part derived from serum proteins. However, the percentage of hexose, hexosamine and sialic acid were considerably higher than values for the total serum proteins.

The presence of serum proteins in urine has been demonstrated immunoelectrophoretically by Grant (1957, 1959) and Patte, Baldassaire & Loret (1958). Two of these components are serum albumin and orosomucoid, which were detected immunochemically in the normal fractions 6–8. In the normal urine fractions, reactions with anti-orosomucoid serum were feeble, indicating a low concentration of urinary orosomucoid and that orosomucoid is not a major glycoprotein of normal urine, although it has been found to be relatively abundant in urines from patients with nephrosis (Popenoe, 1955). The urine-glycoprotein fraction prepared by adsorption on to benzoic acid (Anderson & Maclagan, 1955) is known to be heterogeneous, both on electrophoresis (Biserte, Tayeau, Montreuil, Holleman & Dautrevaux, 1956) and after fractionation on DEAE-cellulose (Kickhöfen *et al.* 1958). It is probable that some of the components of this fraction are of pre-renal origin. The electrophoretic properties at pH 8·6 of fractions 1–8 (Figs. 2 and 4) showed the general trend, observed with the serum proteins, toward higher mobilities in fractions coming off the column at lower pH. These fractions also stained strongly with protein-staining dyes.

The second section (fractions 9–11) contained little ultraviolet-absorbing material but considerable hexose, uronic acid, hexosamine and calcium. These fractions stained very feebly with protein-staining dyes, and were either immobile on electrophoresis at pH 8·6 and 4·6 or had mobilities mainly in the α- and β-globulin range.

The third group (fractions 12–16) absorbed at 280 mμ and contained in addition appreciable amounts of hexuronic acid. Fraction 12 (Fig. 1) had a considerable amount of negatively charged material so far not detected in serum, much of which migrated on paper electrophoresis at pH 4·6 considerably further than serum orosomucoid. Similar fast-migrating material had been noted previously by Markham, Jacobs & Fletcher (1956), who observed two very acidic electrophoretic components in urine, both of which stained more strongly for carbohydrate than for protein. These protein colloids with low isoelectric points may well be included in fraction 12.

The distribution of colloids capable of inhibiting haemagglutination of erythrocytes by virus into a number of widely separable components suggests either that the urinary glycoprotein of Tamm & Horsfall (1950) is easily depolymerized into active sub-units (Porter & Tamm, 1955) or that there are several inhibitory substances in urine. At least two viral haemagglutin inhibitors with differing electrophoretic mobilities have been demonstrated in serum (Burnet, 1951; Tyrrell, 1954), these being immunochemically distinct from the Tamm & Horsfall glycoprotein (Grant, 1957).

There is at present little information about the binding of calcium by urine-colloid fractions. Calcium-binding by various serum proteins, however, has been investigated by many workers. It is generally considered that albumin binds the most calcium (Schmidt & Greenberg, 1935; Prasad & Flink, 1958) and some binding would appear to be related to the presence in serum of proteins of phospholipid (Drinker & Zinsser, 1943).

In this investigation, the more acidic fractions isolated from normal urine (fractions 12–16, Table 3) were the strongest calcium-binders.

The urine colloids from poliomyelitis patients (Fig. 6) bound calcium more strongly than the colloids from a series of normal individuals, although there was no significant difference between the stone-free and stone-forming poliomyelitis urines.

It is unlikely that these poliomyelitis urines, except those exhibiting proteinuria, contain quantitatively more colloidal material than normal urines, since in previous studies (Maclagan & Anderson, 1958*a*, *b*) no significant increase of the urine colloids above normal levels could be detected in a series of patients with renal calculi. It is more likely that a qualitative difference is present, there being an increased excretion of colloidal material in poliomyelitis with strong affinity for calcium.

This work has served to illustrate the wide distribution among the various urine fractions of considerable amounts of carbohydrate-containing colloids of which virtually nothing is known. The evidence presented here would suggest the presence of many glycoprotein entities, some of which, like orosomucoid, are derived from the blood, whereas others, like the glycoprotein isolated by Tamm & Horsfall (1950, 1952), may originate from the urinary tract. The source of the mucopolysaccharides is unknown.

The results presented here suggest that the proteins in urine can be separated on the anion-exchanger DEAE-cellulose. This method, however, does not appear as satisfactory for the separation

of mucopolysaccharides since it is probable that non-electrostatic forces are involved in their retention by the cellulose, most of the mucopolysaccharides being liberated only at very low pH values. Owing to the complexity and diversity of the colloids in urine it is unlikely that an ideal separation will be achieved by the use of any one method, and some combination of several techniques will be required in order to isolate colloids in a homogeneous state.

SUMMARY

1. An ultrafiltration method for recovering the colloids from normal human urine has been presented, and fractionation of these colloids has been carried out on the ion-exchanger DEAE-cellulose, a 2-amino-2-hydroxymethylpropane-1:3-diol–citrate buffer system being used as the eluent, with a gradually descending pH from 9·0 to 1·5. The colloids in each fraction have been isolated by freeze-drying and analysed for hexose, hexosamine, sialic acid, fucose, hexuronic acid, nitrogen and calcium. Their electrophoretic properties at pH 8·6 and 4·6 have also been reported.

2. The ability of these colloid fractions to inhibit the haemagglutination caused by influenza virus has been determined, as has their ability to react with antibodies produced against human-serum albumin and orosomucoid.

3. Fractionation of the urine colloids from two extensively paralysed patients with poliomyelitis, one of whom was forming renal calculi, revealed qualitatively similar chromatographic patterns with certain quantitative differences.

4. The ability of the colloid fractions to bind calcium has been measured by an equilibrium-dialysis technique. Calcium-binding by total non-dialysable urine colloids was found to be significantly above normal in the urine of patients recovering from poliomyelitis. No difference could be detected, however, between the urines from patients who were forming renal calculi and those who were not.

We are very grateful to Dr W. H. Boyce for samples of uromucoid and to Dr B. Weissmann for the preparation of chondroitin sulphate. We wish to thank Professor Otto Westphal and Otto Ludevitz for a generous gift of lipopolysaccharide isolated from *Escherichia coli*. This work was supported by a grant from the National Foundation.

REFERENCES

Anderson, A. J. (1954). *Biochem. J.* **56**, xxv.
Anderson, A. J., Lockey, E. & Maclagan, N. F. (1955). *Biochem. J.* **60**, xli.
Anderson, A. J. & Maclagan, N. F. (1955). *Biochem. J.* **59**, 638.
Baar, S. (1956). *J. clin. Path.* **9**, 144.
Biserte, G., Tayeau, F., Montreuil, J., Holleman, J. & Dautrevaux, N. (1956). *Clin. chim. Acta*, **1**, 115.
Björnesjö, K. B., Werner, I. & Odin, L. (1959). *Scand. J. clin. Lab. Invest.* **7**, 238.
Bonomo, E., Salteri, F. & Cirla, E. (1958). *Arch. Stud. Fisiopat.* **22**, 49.
Boyce, W. H., Garvey, F. K. & Norfleet, C. M. (1954). *J. clin. Invest.* **33**, 1287.
Boyce, W. H. & King, J. S. (1959). *J. clin. Invest.* **38**, 1525.
Boyce, W. H., King, J. S., Little, J. M. & Artom, C. (1958). *J. clin. Invest.* **38**, 1658.
Boyce, W. H. & Swanson, M. (1955). *J. clin. Invest.* **34**, 1581.
Burnet, F. M. (1951). *Physiol. Rev.* **31**, 131.
Craddock, J. C. & Kerby, G. P. (1955). *J. Lab. clin. Med.* **46**, 193.
DiFerrante, N. & Rich, C. (1956). *Clin. chim. Acta*, **1**, 519.
Dische, Z. (1947). *J. biol. Chem.* **167**, 189.
Dische, Z. & Shettles, L. B. (1948). *J. biol. Chem.* **175**, 595.
Drinker, N. & Zinsser, H. H. (1943). *J. biol. Chem.* **148**, 187.
Durram, E. L. (1950). *J. Amer. chem. Soc.* **72**, 2943.
Einbinder, J. & Schubert, M. (1951). *J. biol. Chem.* **191**, 591.
Faillard, H. (1956). *Hoppe-Seyl. Z.* **305**, 145.
Ferro, P. & Hamm, A. B. (1957). *Amer. J. clin. Path.* **28**, 689.
Flynn, F. V. & De Mayo, P. (1951). *Lancet*, ii, 235.
Goodman, M., Ramsey, S. S., Simpson, W. L., Ranip, D. G., Basinski, D. H. & Brennan, M. J. (1957). *J. Lab. clin. Med.* **49**, 151.
Grant, G. H. (1957). *J. clin. Path.* **10**, 360.
Grant, G. H. (1959). *J. clin. Path.* **12**, 510.
Hammerman, D. & Hatch, F. T. (1955). *Proc. Soc. exp. Biol., N.Y.*, **89**, 279.
Heremans, J. (1958). *Clin. chim. Acta*, **3**, 34.
Jacques, L. B., Bapke, E. & Levy, S. W. (1953). *Circulation Res.* **1**, 321.
Kabat, E. A. & Meyer, M. M. (1948). *Experimental Immunochemistry*. Springfield, Ill.: Charles C. Thomas Publisher.
Kerby, G. P. (1954). *J. clin. Invest.* **33**, 1168.
Kickhöfen, B., Struwe, F. E., Bramesfeld, B. & Westphal, O. (1958). *Biochem. Z.* **330**, 467.
King, J. S., Boyce, W. H., Little, J. M. & Artom, C. (1958). *J. clin. Invest.* **37**, 315.
King, J. S., Little, J. M., Boyce, W. H. & Artom, C. (1959). *J. clin. Invest.* **38**, 1520.
Laurent, B. (1958). *Scand. J. clin. Lab. Invest.* **10**, Suppl. 32.
Lockey, E., Anderson, A. J. & Maclagan, N. F. (1956). *Brit. J. Cancer*, **10**, 209.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
McGarry, E., Schon, A. J. & Rose, B. (1955). *J. clin. Invest.* **34**, 832.
Maclagan, N. F. & Anderson, A. J. (1958*a*). *Brit. J. Urol.* **30**, 269.
Maclagan, N. F. & Anderson, A. J. (1958*b*). *Ciba Foundation Symp., The Chemistry and Biology of Mucopolysaccharides*, p. 268.
Markham, R. L., Jacobs, J. H. & Fletcher, E. T. D. (1956). *J. Lab. clin. Med.* **48**, 559.

Möerner, A. H. (1895). *Skand. Arch. Physiol.* **6**, 332.
Patte, J. C., Baldassaire, G. & Loret, J. (1958). *Rev. franç. Étud. clin. biol.* **3**, 960.
Peterson, H. A. & Sober, E. A. (1958). *Fed. Proc.* **17**, 288.
Popenoe, E. A. (1955). *J. biol. Chem.* **217**, 61.
Porter, K. R. & Tamm, I. (1955). *J. biol. Chem.* **212**, 135.
Prasad, A. S. & Flink, E. B. (1958). *J. Lab. clin. Med.* **51**, 345.
Rich, C. & DiFerrante, N. (1956). *Clin. Chem.* **2**, 235.
Rigas, D. A. & Heller, C. G. (1951). *J. clin. Invest.* **30**, 853.
Rondle, C. J. M. & Morgan, W. T. J. (1955). *Biochem. J.* **61**, 586.
Schmidt, C. L. A. & Greenberg, D. N. (1935). *Physiol. Rev.* **15**, 297.
Shatton, J. & Schubert, M. (1954). *J. biol. Chem.* **211**, 565.
Sober, H. A., Cutter, F. J., Wyckoff, M. M. & Peterson, E. A. (1956). *J. Amer. chem. Soc.* **78**, 756.
Sober, H. A. & Peterson, E. A. (1958). *Fed. Proc.* **17**, 1116.
Sonnet, J. (1956). *Rev. belge. Path.* **25**, 498.
Tamm, I., Bugher, J. C. & Horsfall, F. L. (1955). *J. biol. Chem.* **212**, 125.
Tamm, I. & Horsfall, F. L. (1950). *Proc. Soc. exp. Biol., N.Y.*, **74**, 108.
Tamm, I. & Horsfall, F. L. (1952). *J. exp. Med.* **95**, 71.
Tyrrell, D. A. J. (1954). *J. Immunol.* **72**, 494.
Werner, I. & Odin, L. (1952). *Acta Soc. Med. Upsalien.* **57**, 230.
Williams, C. A. & Grabar, P. (1955). *J. Immunol.* **74**, 158.
Winzler, R. J. (1955). *Meth. biochem. Anal.* **2**, 279.

Biochem. J. (1960) **77**, 591

The Mode of Recovery of Cholinesterase Activity *in vivo* after Organophosphorus Poisoning

1. ERYTHROCYTE CHOLINESTERASE

BY L. C. BLABER* AND N. H. CREASEY
Chemical Defence Experimental Establishment, Porton Down, Salisbury, Wilts.

(*Received* 6 *April* 1960)

It is now generally accepted that the inhibition of cholinesterase by organophosphorus compounds consists of a direct phosphorylation of the active centre with the formation of a disubstituted phosphoryl cholinesterase (Aldridge, 1953). If the inhibited enzyme is kept *in vitro* in the absence of excess of inhibitor, a certain amount of spontaneous reactivation occurs by hydrolysis of the phosphorylated enzyme, the exact amount being dependent on the groups attached to the phosphorus atom (Aldridge, 1953; Burgen & Hobbiger, 1951; Aldridge & Davison, 1953).

Reactivation of the inhibited cholinesterase can be markedly accelerated by treating it with certain nucleophilic reagents such as choline (Wilson, 1951), hydroxamic acids (Wilson, 1955) and oximes (Childs, Davies, Green & Rutland, 1955; Wilson & Ginsberg, 1955; Davies & Willey, 1957), the rate and extent of reactivation again being dependent on the groups attached to the phosphorus atom. The oximes are by far the best reactivators and under suitable conditions, with freshly inhibited enzyme, oxime treatment will restore all the enzyme activity (Davies & Green, 1956).

If the freshly inhibited and completely reactivatable cholinesterase is stored it gradually changes into a form which can no longer be reactivated by oximes (Davies & Green, 1956; Wilson, Ginsberg & Meislich, 1955; Hobbiger, 1955; Hobbiger, 1956; Jandorf, Michel, Schaffer, Egan & Summerson, 1955). This may be due either to the migration of the phosphoryl group to a more stable position in the inhibited enzyme (Wilson, Ginsberg & Meislich, 1955; Hobbiger, 1956; Jandorf *et al.* 1955), or to loss of one of the substituents on the phosphorus atom which would then acquire a negative charge and consequently become resistant to nucleophilic attack (Oosterbaan, Warringa, Jansz, Berends & Cohen, 1958). The change which occurs during storage will be referred to here as 'aging' although some authors have in fact referred to it as a transphosphorylation process (Hobbiger, 1956; Jandorf *et al.* 1955).

Little is known about the extent to which these reactions influence the *in vivo* recovery of inhibited cholinesterase. Davison (1955) suggested that the return to normal levels of activity was probably the combined result of spontaneous hydrolysis of the inhibited enzyme and of new enzyme synthesis. He found that after inhibition *in vivo* by diethyl *p*-nitrophenyl phosphate (E 600) the activities of rat-brain cholinesterase and of a variety of erythrocyte cholinesterases returned to normal in two

* Present address: School of Pharmacy, Department of Pharmacology, P.O. Box 125, 29/39 Brunswick Square, London, W.C. 1.