The Precipitation of Polyanions by Long-Chain Aliphatic Ammonium Salts

6. THE AFFINITY OF SUBSTITUTED AMMONIUM CATIONS FOR THE ANIONIC GROUPS OF SOME BIOLOGICAL POLYMERS*

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Polyanions are quantitatively precipitated from aqueous solution by cationic detergents such as cetylpyridinium chloride (Scott, 1955, 1960). The water-insoluble complexes are soluble in salt solutions, provided that the salt concentration is not below a certain critical level, the critical salt concentration. The value of the critical salt concentration depends on the nature of the salt (Scott, 1955), the structure and molecular weight of the polyanion (Scott, 1955, 1956; Laurent, Ryan & Pietruszkiewicz, 1960), and the chain length (Scott, 1961) and cationic group of the detergent. It is, however, a constant for the polyanion, if the salt and cationic detergent are defined, and this finding has been applied to the fractionation of polyanion mixtures of biological origin [see, for example, Scott (1960)].

In the hope of improving and elucidating the fractionation procedures, the present investigation was carried out to determine the effect on the critical salt concentration of the cationic group of the detergent (including primary, secondary, tertiary and quaternary ammonium compounds, and pyridinium and guanidinium salts). In making this comparison one is probably assessing the relative affinity of variously substituted cationic nitrogen for the negative groups of the polyanions. The results are therefore of interest in a wider sphere, since such cationic groups are found in many dyes, drugs, antiseptics and polycations of biological importance, and their biological activity may depend primarily on the possession of a cationic nitrogen function.

EXPERIMENTAL

Materials

Stock solutions were prepared as follows and diluted as needed. Except where stated, the chemicals were laboratory reagent grade.

Amines. Solutions (approx. 5M) of ammonium chloride (AnalaR), methylammonium chloride and dimethylammonium chloride (British Drug Houses Ltd.) were pre-

pared from the solid. Trimethylammonium chloride (5M) was prepared from the commercial 50% (w/v) solution (British Drug Houses Ltd.). Each pH was checked, and adjusted to 6.0–6.5 with a few drops of *N*-NaOH. Tetramethylammonium chloride (1.9M) was made by neutralizing commercial 25% (w/v) tetramethylammonium hydroxide solution (British Drug Houses Ltd.) to pH 6.0 with 5*N*-HCl. Guanidinium hydrochloride (4M) was prepared by dissolving the theoretical quantity of AnalaR guanidinium carbonate (Hopkin and Williams Ltd.) in 5*N*-HCl, and warming to drive off dissolved CO₂. The pH was adjusted to 6.5 with a few drops of *N*-HCl. The molarities of the above hydrochloride solutions were based on the chloride content as determined by the method of Schales & Schales (1941). Cetylpyridinium chloride (pure) (1%, w/v) (L. Light and Co. Ltd., Colnbrook, Bucks.) and *N*-cetyltrimethylammonium bromide (1%, w/v) (British Drug Houses Ltd.) were prepared from the solid. *N*-Cetylmethylammonium chloride [1%, w/v (approx.)] was prepared by dissolving 0.5 g. of cetylmonomethylamine (a gift from the Imperial Chemical Industries Ltd. specimen collection) in slightly less than the equivalent amount of 0.1*N*-HCl with warming, and adjusting to pH 5.9 with 0.1*N*-HCl, in a volume of 50 ml. *N*-Cetyldimethylammonium chloride [1% (approx.)] was prepared by dissolving 1.1 ml. of DM16D (cetyldimethylamine; a gift of Armour Hess Ltd., 6 Arlington Street, St James's, London, S.W. 1), in 19.0 ml. of 0.1*N*-HCl with warming, and adjusting to pH 5.7 in a volume of 100 ml.

N-Dodecyltrimethylammonium bromide (pure) (1%, w/v) (Leda Chemicals Ltd., Berk House, Portman Square, London, W. 1) was prepared from the solid. Dodecyltrimethylammonium chloride [1%, w/v (approx.)] was prepared by dissolving approximately 1 ml. of DM12D (*N*-dodecyltrimethylamine; a gift of Armour Hess Ltd.) in 31.5 ml. of 0.1*N*-HCl, and adjusting the pH to 6.0, in a volume of 100 ml. Tetradecylammonium chloride (1%, w/v) was prepared by dissolving the solid hydrochloride made as described by Scott (1961) from the free amine (L. Light and Co. Ltd.) with warming to 30°.

Polyanions. These were: sodium hyaluronate (0.1%), prepared from hyaluronic acid isolated from human mesothelioma fluid by the method of Scott (1960); sodium alginate (0.1%), British Pharmaceutical Codex grade (Hopkin and Williams Ltd.); sodium deoxyribonucleate (DNA) (0.1%) prepared from DNA (highly polymerized, Worthington Biochemical Corp., N.J., U.S.A.); heparin (0.1%), diluted from Pularin (Evans Biological Chemicals Ltd., Runcorn, Cheshire) (20 mg./ml.).

* Part 5: Scott (1961).

Methods

Determination of the critical salt concentration. This was determined in a water bath at 30°, except where otherwise indicated. Wherever possible the solubility of the precipitate in the salt solution (sodium or substituted ammonium chlorides) was followed by measuring the ultraviolet (u.v.) absorption of the polyanion (DNA) or precipitant (cetylpyridinium ion) in the supernatant. The results agreed well with a semiquantitative assessment made by eye. Where neither the polyanion nor the detergent absorbed light the disappearance of the precipitate at the critical salt concentration was followed solely by eye. The basic procedure was as follows (Scott, 1955).

Polyanion solution (0.5 ml.) containing 0.5 mg. of polyanion was added to each of a series of 5 ml. conical centrifuge tubes and mixed with salt solution and water to 3.5 ml. The proportions of stock concentrated salt solution and water were calculated to give a stepwise increase of salt concentration along the series. Cetylpyridinium chloride solution (0.5 ml. of 0.4%) was added, the precipitates were left to form for 2–4 hr., and centrifuged at 1000*g* for 5 min. A portion (1 ml.) of each supernatant was diluted with 3 ml. of M-KCl solution and the extinction at 260 μ was determined. Supernatant solutions from experiments with heparin were diluted 1:5 with 1.3M-KCl. Controls were included in each series to correct for u.v. absorption by the salt or polyanion.

An alternative procedure, used when determining critical salt concentrations for sodium alginate or hyaluronate with mixtures of amines, was as follows: sodium alginate (1 ml. of 0.25%) or sodium hyaluronate (0.20%) was added to 2 ml. of 2M-NaCl, or 2 ml. of 1M-NaCl, respectively, in a test tube at 30° in a water bath. Amine solution [1 ml. of 1% (w/v)] was added followed by water, slowly with careful mixing, until the polyanion-amine precipitate appeared and completely flocculated. The critical

salt concentration was taken as that concentration of sodium chloride at which there was no increase in flocculent precipitate on further addition of water. The results are therefore lower limits.

RESULTS

The critical salt concentrations in sodium chloride of the polyanion-amine complexes are given in Table 1. It was necessary to use a temperature of 60° for the comparison of members of the cetyl series because of the insolubility of the hydrochlorides at lower temperatures, especially in the presence of sodium chloride. The dodecyl compounds were also compared at 60°, and as many members of both series as solubility limitations would allow were investigated at 30°. Table 1 shows that the order in each series is unaffected by temperature changes, and the actual values are only slightly increased as the temperature rises from 30° to 60°. A series of substituted ammonium ions without long chains was included to determine whether the presence of a long chain had any effect on the 'affinity' of the cationic 'head' for the polyanion and to throw some light on the observed differences. The critical salt concentration of the cetylpyridinium complex of each polyanion was determined in the appropriate amine hydrochloride solution, and the results are shown in Figs. 1 (a)–(c). Measurements were also made in guanidinium chloride, because of the biological interest of substituted guanidine, and as a possible guide to the behaviour of long-chain alkylguanidinium salts. Table 2 includes results obtained with

Table 1. *Critical salt concentrations in sodium chloride solution of substituted cetylammmonium and dodecylammmonium complexes of heparin and alginate*

The basic procedure (see Methods section) was used to determine critical salt concentrations. The disappearance of the precipitates was followed by eye. The critical salt concentrations are expressed as molarities.

	Heparin		Alginate	
	60°	30°	60°	30°
Cetylpyridinium chloride	1.65	1.55	0.40	0.38
Cetyltrimethylammonium bromide	1.05	1.00	—	—
Cetyltrimethylammonium chloride	1.75	—	—	—
Cetyltrimethylammonium chloride	2.40	—	—	—
Dodecylpyridinium chloride	1.35	1.25	—	—
Dodecyltrimethylammonium chloride	0.95	0.90	—	—
Dodecyltrimethylammonium chloride	1.40	1.35	—	—

Table 2. *Critical salt concentrations in solutions of substituted ammonium chlorides of hyaluronic acid and dextran sulphate complexes of cetylpyridinium*

The basic procedure (see Methods section) was used to determine critical salt concentrations, which are expressed as molarities.

	NH ₄ ⁺	MeNH ₃ ⁺	Me ₂ NH ₂ ⁺	Me ₃ NH ⁺	Me ₄ N ⁺
Dextran sulphate*	1.75	2.05	2.5	—	—
Hyaluronic acid	0.235	0.245	0.26	0.275	0.275

* Results from Scott (1955).

hyaluronic acid and dextran sulphate, those for the latter being taken from Scott (1955).

In the experiments with DNA the degree of precipitation of the polycation was followed by direct measurement at 260 $m\mu$. Cetyltrimethylammonium bromide was used as the precipitant instead of cetylpyridinium chloride, to avoid interference from the strong u.v. absorption of cetylpyridinium ion, which is entirely absent from cetyltrimethylammonium ion. The solution of guanidinium

hydrochloride had a considerable extinction at 260 $m\mu$, in the concentrations used in the DNA experiment, and the readings were therefore carried out at 280 $m\mu$, where the interference was much smaller, and the results were corrected to 260 $m\mu$ for comparison with the other members of the series.

Because of the insolubility of long-chain primary ammonium hydrochlorides in concentrated sodium chloride solutions, a direct determination of the critical salt concentration could not be made, but use was made of the fact that their solubility considerably increased in the presence of quaternary amines (such as cetyltrimethylammonium bromide). Tetradecylammonium chloride (1%, w/v) and cetyltrimethylammonium bromide (1%, w/v) were mixed in the ratios shown in Table 3. It was not possible to increase the ratio of tetradecylammonium to cetyltrimethylammonium beyond 3:2, because of the appearance of heavy precipitates of the amine hydrochloride in the concentrated salt solutions. The results shown in Table 3 include only those in which the appropriate control solution in the absence of polyanion was essentially free of precipitated amine. The results of the alginate experiment fit well on a plot of the log of the critical salt concentration against percentage of primary amine in the mixture (Fig. 2). Extrapolation of the line to 100% of primary amine gives a value of about 4.5–4.7M for the critical salt concentration. The logarithmic plot is not quite so successful with hyaluronate.

Fig. 1. Plots of the extinction at 260 $m\mu$ of the supernatants of precipitated complexes of (a) heparin and (b) alginate with cetylpyridinium ion, and (c) DNA with cetyltrimethylammonium ion, as a function of salt concentration. An increase in extinction coincides with an increase in solubility of the complex in the salt solution. Precipitation is complete at the left-hand side of the graph, where extinctions are lowest, and solubility is complete at the highest extinctions. The basic procedure (see Methods section) was used in these experiments, except that the extinction of supernatants from experiments involving guanidinium chloride and DNA were determined at 280 $m\mu$ and corrected to 260 $m\mu$. ▲, Ammonium chloride; ○, methylammonium chloride; △, dimethylammonium chloride; □, trimethylammonium chloride; ■, tetramethylammonium chloride; ●, guanidinium chloride.

Table 3. *Critical salt concentrations in sodium chloride solution of hyaluronate and alginate complexes with mixtures of tetradecylammonium chloride and cetyltrimethylammonium bromide*

The alternative procedure (see Methods section) was used to determine critical salt concentrations, which are expressed as molarities. The figures in columns 1 and 2 represent the volumes of each solution in 100 ml. of a mixture of the two.

1% (w/v) Tetradecylammonium chloride (ml.)	1% (w/v) Cetyltrimethylammonium bromide (ml.)	Critical salt concentrations in NaCl	
		Sodium hyaluronate	Sodium alginate
	00	0.204	0.27
20	80	0.248	0.50
40	60	0.483	0.84
60	40	0.745	1.50
80	20	—	>2.7

Fig. 2. Plot of $[1 + \log (\text{critical salt concentration})]$ against the percentage composition of the long-chain amine precipitant: TDAC, tetradecylammonium chloride; CTAB, cetyltrimethylammonium bromide. The alternative procedure (see Methods section) was used to determine the critical salt concentration in sodium chloride at which the hyaluronate (○) or alginate (●) complex dissolved.

DISCUSSION

Three types of ionized groups are present in the polyanions studied: ester sulphate, diester phosphate and carboxylate. These are the only anionic groups that have been found in quantity in biological polymers. The existence of striking differences in the affinity of inorganic cations for these groups is the most important factor in the fractionation procedures referred to, and, if analogous preferences could be discovered among the cationic groups of detergents, a large increase in flexibility would be expected in the techniques of fractionation.

The results of this investigation are unequivocal; there is a marked decrease in the critical salt concentration as the degree of methylation of the amine nitrogen increases, whether the anionic

charge is due to carboxyl or sulphate groups (Tables 1 and 3). This does not offer the desired opportunity to exploit a specific type of interaction. There is a suggestion, however, that the guanidinium ion may stand in a different relationship to the ammonium series when a polyphosphate is involved [Fig. 1 (a)].

A general increase in the values (such as would result on replacing, e.g. cetyltrimethylammonium bromide with cetylmonomethyl ammonium chloride) expands the range in which the critical salt concentrations occur, and may be of use in situations in which they are at present closely grouped. The increased range is not obtained without some disadvantages: the long-chain secondary methylamines are difficult to prepare, the primary amines give few soluble salts at room temperature, and the corresponding polyanion complexes are rather insoluble in salt solutions and organic solvents. They are all much weaker bases than the quaternary bases, which limits the range of pH in which they may be used. Similar disadvantages are associated with the long-chain alkyl guanidinium salts, e.g. dodecylguanidinium acetate (Cyprex), except that they are strong bases.

As the proportion of primary ammonium ion in a mixture with quaternary ammonium salt increases, there is a corresponding marked increase in the critical salt concentration of the complexes formed in the presence of the mixture (Table 3). The difference in the values for hyaluronate and alginate, which is about 0.1M-NaCl, when cetyltrimethylammonium is the precipitating ion, is increased to about 0.75M in a 3:2 mixture of tetradecylammonium chloride and cetyltrimethylammonium bromide. Separation of similar polyanions might therefore be improved by mixing primary and quaternary amines in the fractionation system. For reproducible results it would probably be necessary to have a large excess of precipitant, since the composition of the amine in the precipitate does not necessarily reflect the relative proportions in the precipitant, and there may be a comparative enrichment of the quater-

nary amine in the supernatant (J. E. Scott, unpublished work).

In general it appears that, as the cationic nitrogen of the detergents becomes more encumbered with substituent groups, the critical salt concentration decreases. The considerable drop on passing from alkylpyridinium to alkyltrimethylammonium ion is especially interesting, since both nitrogens are quaternary; but, whereas the alkyltrimethylammonium nitrogen is entirely surrounded by substituent groups arranged tetrahedrally, the pyridinium nitrogen is a member of a planar mesomeric ring system, with presumably good access from above and below the plane of the ring.

Scott (1961) has suggested that the basis of the occurrence of a critical concentration of salt may be a competition between the detergent ion and the inorganic ion. If this is so, it may provide a way of arranging the competing ions in the order of their affinities for the charged groups of the polyanion. If it is assumed that their affinities are inversely proportional to the concentrations at which they displace the reference ion (e.g. cetylpyridinium), the following descending order of affinities may be deduced from the experimental data: NH_4^+ , Me_2NH_3^+ , Me_3NH_2^+ , Me_3NH^+ , Me_4N^+ ion. The same sequence is obtained if the groupings are part of a detergent molecule (Table 1), where Na^+ is the reference ion. The result is not affected by the method of estimation (i.e. by eye, or by following the u.v. absorption of the polyanion or of the detergent cation).

Teunissen-Van Zÿp [quoted by Bungenberg de Jong (1949)] has concluded that the affinities are in exactly the reverse sequence. This conclusion was based on studies of the electrophoretic mobility of polycarboxylates and polyphosphates in solutions of methylated ammonium chlorides. As the hydrochloride concentration was increased the mobility of the polyanion decreased to zero, and then reversed in direction. The concentration of hydrochloride at the reversal point was supposed to be directly proportional to the cation-polyanion affinity. The discrepancy between the two sets of results is the more interesting, since in every other case so far investigated by both techniques there has been close agreement [e.g. comparison of the 'ion spectra' of Bungenberg de Jong (1949) with the critical salt concentration sequences reported by Scott (1955)].

The conclusion of Gregor, Gold & Frederick (1957), based on viscometric and conductimetric studies, that Na^+ ion is more firmly bound than tetramethylammonium and other tetra-alkylammonium ions to polymethacrylate, is compatible with the present findings, which show a higher critical salt concentration for the cetylpyridinium salt of alginic acid in tetramethylammonium chloride than in sodium chloride: 0.65 [Fig. 1(b)]

as compared with 0.38 (Table 1). It is not easily reconcilable with the results of Bungenberg de Jong on sodium pectinate, which suggest that the Na^+ ion is less strongly bound, in view of the 20-fold increase in the reversal of charge concentration in sodium chloride [Bungenberg de Jong (1949), p. 284] as compared with tetramethylammonium chloride [Bungenberg de Jong (1949), p. 304]; this assumes that only the nature of the acid group is important in the binding of cations.

The efficiency of the precipitation of polyanions by quaternary ammonium detergents is less than that of primary ammonium detergents (Scott, 1961), which is consistent with a greater affinity of the latter for the anionic groups.

However, to make a valid comparison of affinities it is necessary to know the activities of the competing ions. The data for substituted ammonium ions are meagre, as they are for cetylpyridinium ion in salt solutions. The application of the existing data for the amine hydrochlorides [quoted by Robinson & Stokes (1959)] would diminish the observed differences considerably, since there is a decrease along the series from NH_4^+ to Me_4N^+ ion. The corresponding corrections for the activity of cetylpyridinium ion in the various concentrations of chloride cannot be made at present. The activity of sodium chloride at 30° diminishes slightly from 0.5 to 2.5M. If the activities of the long-chain amine hydrochlorides decrease along the series from RNH_3^+ to RNHMe_2^+ ion to the same extent as those of the simple amine hydrochlorides, the observed differences (Table 1) would be reduced.

There are five possible situations in the type of experiment described here, if the activity of the reference ion is assumed to change insignificantly within each series, and if the anion is chloride. The affinity for the polyanion of the substituted ammonium cations, for the polyanion taken along the series from NH_4^+ to Me_3NH^+ ion, may (a) decrease, (b) remain the same, or increase; the increase may be (c) less rapid, (d) at the same rate, or (e) more rapid than the increase in the affinity for chloride. The increase in affinity for the Cl^- ion is inferred from the drop in activity coefficient from NH_4^+ to Me_3NH^+ ion.

Possibilities (a), (b) and (c) should result in the same experimental finding, that the critical salt concentration increases from NH_4^+ to Me_3NH^+ ion, which is the result obtained in this investigation. The experiments given here do not differentiate further between the three. The results of Teunissen-Van Zÿp, referred to above, tend to eliminate (a) and (b), leaving (c) as apparently the only basis on which both sets of experiments can be interpreted qualitatively. It is, however, the most difficult to

reconcile quantitatively with the present results, since it would be expected to lead to smaller differences than are found. A part of the discrepancy may be due to small changes in the activity of the reference ion with changes in chloride concentration.

The results are as characteristic of the Cl^- anion as they are of the other participants. If another anion had been chosen such that the activity coefficients of the substituted ammonium salts had increased, rather than decreased, from NH_4^+ to Me_3NH^+ ion, the present series could possibly have been considerably changed, if not entirely reversed. The competing cations can be considered as being 'partitioned', in a sense, between the polyanion and the ambient anion, the balance being decided by their relevant affinities.

SUMMARY

1. The concentration of sodium chloride needed to dissolve complexes of the polyanions heparin, deoxyribonucleate, hyaluronate, alginate and dextran sulphate with long-chain aliphatic ammonium detergents decreased with increasing degrees of methylation of the cationic nitrogen.

2. The concentration of ammonium chloride and its methyl derivatives needed to dissolve the cetyl-

pyridinium complexes with the same polyanions increased with increasing degrees of methylation of the cationic nitrogen. Guanidinium chloride and ammonium chloride were comparable except that the deoxyribonucleate complex dissolved in a more dilute solution of the former.

3. It is assumed that there is competition between the cations of solvent salt and detergent, and the results are discussed on this basis.

4. The relevance of these results to systems in which there is binding to polyanions of substituted ammonium compounds is discussed.

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Liver Lipids and Ketone-Body Formation in Rats Deficient in Pantothenate

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Pantothenic acid is a constituent of coenzyme A (Lipmann, Kaplan, Novelli, Tuttle & Guirard, 1947; Novelli, Kaplan & Lipmann, 1949), and deficiency of this dietary factor has been shown to lower the concentration of the coenzyme in the tissues. Olson & Kaplan (1948) reported an immediate effect of deficiency of the vitamin on the concentration of the coenzyme in the adrenal of the rat, followed later by a 35–40% decrease in kidney, heart and liver. Such changes are associated with widespread disturbances of metabolism. Thus decrease of the concentration of the coenzyme in the cardiac muscle of ducklings (Olson & Stare, 1951), and of the liver of ducks and rats (Olson &

Kaplan, 1948) results in an impaired utilization of pyruvate.

There is also evidence of an impairment of acetate metabolism in pantothenate deficiency. Klein & Lipmann (1953*a*), using slices of rat liver incubated with carboxyl-labelled acetate, observed a marked decrease in the rate of synthesis of fatty acids and of cholesterol after 6 weeks of deprivation of the vitamin. Klein & Lipmann (1953*b*) also demonstrated a correlation between the concentration of coenzyme A and the capacity to synthesize lipid and cholesterol in *Saccharomyces cerevisiae* grown in pantothenate-deficient media.

Pantothenic acid has therefore been implicated in various aspects of lipid metabolism. The present paper reports observations on the analysis of liver

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