Influence of Cosolutes upon the Conformation of Carbohydrates in Aqueous Solutions. Part III.[1] Effect of Inorganic Ions upon the Binding of Iodine by Starch and Derivatives of Amylose

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For solutions of starch, diethylaminoethylamylose, amylose sulphate, and carboxymethylamylose in aqueous inorganic acids or salts at 20°, plots of the optical density at 680 nm against the concentration of added potassium tri-iodide (KI_3) showed maxima whose values were highly dependent upon the concentration and identity of the ions present. The absorption spectra in the different media were also different, and similar changes in the absorption spectra could not be brought about by varying the concentration of I_3^- in the system.

With polyvinyl alcohol, there was no tendency to develop different maxima in different media, and the absorption spectrum was independent of the medium. It thus appeared that the acids and salts were bringing about a conformational change in the glucans, leading to an increase or decrease in the number of sites that were able to bind I_3^- strongly.

By working with a very large excess of I_3^-, it was possible to avoid complications arising from the competitive binding of species other than I_3^-, or salt effects upon the activity coefficient of I_3^-, and to study the effect of different media upon the conformational change alone. These media included aqueous sulphuric, phosphoric, hydrochloric, and hydrobromic acids, alkali-metal halides, and various other salts of lithium, sodium, and potassium.

The effect of the acids and salts upon the maximal optical density at 680 nm could not be correlated with their effect upon the volume of gels prepared by cross-linking starch with epichlorohydrin, or with their salting-out parameters for non-polar compounds. This was interpreted as evidence that the conformational changes were not primarily associated with changes in the stability of intramolecular hydrogen bonding, or of hydrophobic interactions.

On the other hand, there was a close correlation between the maximal optical density in the four acids named above, and their previously demonstrated capacity to change the magnitude of the anomeric effect in the methyl glucopyranosides and in cellobiose and maltose. With an apparent exception that was noted for fluorides, the effect of all the other salts upon the optical density correlated well with their expected capacity to modify the magnitude of the anomeric effect, based upon the idea that the ring and glycoside-oxygen atoms are selectively desolvated by salts whose anion is the stronger electrostrictor, and solvated by salts whose cation is the stronger electrostrictor.

It was therefore concluded that the conformational changes were mainly due to changes in the magnitude of the *exo*-anomeric effect, which would be expected to modify the relative stabilities of the rotamers generated about the C(1)–O(1) bonds, and, hence, the ease with which the linear segments of the glucan chains are able to take up the helical, *V*-conformation in solution. It is pointed out that similar changes can be expected in any flexible molecule, whose net dipole moment in different conformational states can be expected to vary.

In Parts I [2] and II [1], evidence was obtained that inorganic ions, in aqueous solution, have the ability to change the magnitude of the anomeric effect in *O*-glycopyranosides. The results were consistent with the idea that they do this by orientating the water molecules so as to enhance or impair their capacity to form hydrogen bonds with the ring and glycoside oxygen atoms of the glycoside.[1]

This paper is concerned with a direct corollary of these findings, namely, that inorganic ions should also change the magnitude of the *exo*-anomeric effect.[3] This implies that they should change the population of rotamers about the C(1)–O(1) bond, and, hence, the conformation of polysaccharides in aqueous solution.

To test this idea, the effect of inorganic acids and salts upon the iodine-binding capacity of starch and soluble derivatives of amylose has been studied, in an attempt to determine to what extent the observed changes are consistent with those expected on the assumption that the magnitude of the *exo*-anomeric effect is being affected.

THEORY

This section briefly summarises the relevant conclusions reached in Parts I [2] and II,[1] and explains the theoretical basis of the present experimentation.

Water Orientation Hypothesis.—Lemieux and his coworkers [3] have shown that protic solvents have a much stronger capacity to diminish the magnitude of the anomeric effect in *O*-glycopyranosides than aprotic solvents of the same dielectric constant, and they inferred that this was because they are able to form hydrogen bonds with the ring and glycoside oxygen atoms. Water was shown to have an exceptionally strong capacity to quench the anomeric effect.[3]

In order to understand why inorganic ions, in aqueous solution, are able to increase or decrease the magnitude of the anomeric effect,[1,2] it was necessary to consider in what way they could decrease or increase the capacity of water to solvate the ring and glycoside oxygen atoms. The following hypothesis was based upon generally accepted facts about the influence of ions upon the structure of water.[1]

Inorganic ions are usually hydrated in aqueous solution, and the water molecules in the respective hydration shells are orientated, with their hydrogen atoms pointing towards anions, and their oxygen atoms pointing towards cations. For closed-shell ions, like the alkali metals or the halides, the mechanism is simply dipole attraction, and the extent of the orientation, or ' electrostriction,' is inversely related to the ionic radius. An anion should be a stronger electrostrictor than a cation of the same charge and radius, because the smaller size of a hydrogen atom allows it to

[1] Part II, T. J. Painter, *Acta Chem. Scand.*, 1973, **27**, 3839.
[2] T. J. Painter, *Acta Chem. Scand.*, 1973, **27**, 2463.
[3] R. U. Lemieux, A. A. Pavia, J. C. Martin, and K. A. Watanabe, *Canad. J. Chem.*, 1969, **47**, 4427.

get closer to an anion than an oxygen atom can get to a cation.

The water molecules around an anion would be expected to have a diminished capacity to solvate the ring and glycoside oxygen atoms of a glycoside, because they would have to be re-orientated, against the electrostatic field of the anion, in order to do so. On the other hand, the water molecules around a cation would be orientated in such a way as to enhance such hydrogen bonding. In addition to the effect of orientation, the electrostatic field of an ion would be expected to bring about an enhanced polarisation of the O–H bonds in the electrostricted water molecules. This implies that the water molecules around a cation should be stronger proton donors, and those around an anion should be stronger proton acceptors, than ordinary water molecules.

The net effect of any salt, acid, or alkali upon the hydrogen-donating capacity of water should be determined by the ion which is the stronger electrostrictor. However, the effect will not be a simple function in the total concentration of solute, because, as the concentration increases, the anions and cations start to neutralise one another's electrostatic fields, and the hydration shells start to break down. In general, the effect of the stronger electrostrictor should pass through a maximum as the concentration increases. This has been demonstrated experimentally for hydrobromic acid.[1]

An important corollary of this is, perhaps, that one should not always attempt to understand the role of a large ion in terms of any direct effect that it may have upon the structure of water, such as ' structure-breaking.' [4] Because it is unable to approach its counterion very closely, a large ion should ' allow ' it, for a given concentration, to affect the structure of water more profoundly. A very large ion may possibly allow its counterion to behave in much the same way as it would do at infinite dilution. We shall suggest that it is necessary to invoke this idea in order to understand the influence of different cations upon the conformation of anionic polysaccharides in aqueous solution.

Population of Rotamers about the C(1)–O(1) *Bond.*—The three rotamers (A_1—A_3) for methyl α-D-glucopyranoside, and the three (E_1—E_3) for the corresponding β-anomer are shown in Figure 1. For the α-anomer, A_3 would be virtually precluded because of the strong interactions between the methyl group and C(3), H(3), C(5), and H(5), as well as the *gauche* interactions with C(2) and O(5). In A_2, the methyl carbon atom would be in *gauche* interaction with C(2) and H(1), and in opposition to O(2); while in A_1, it would be in *gauche* interaction with O(5) and H(1) only.

For the β-anomer, the methyl carbon atom would be in *gauche* interaction with O(5) and H(1) only in E_1; in *gauche* interaction with C(2) and O(5), and in opposition to H(2) in E_2; and in *gauche* interaction with C(2) and H(1), and in opposition to O(2) in E_3.

The available estimates [5,6] of non-bonded interaction energies are not sufficiently accurate to permit exact calculation of the populations of rotamers that would be expected on the basis of these considerations. It is realistic, however, to work to the nearest kcal, and to conclude that, *in the absence of electronic effects*,* A_1 and E_1 would both be preferred to the extent of 2 kcal mol^{-1}. For the corresponding mannopyranosides and 2-deoxyglucopyranosides, this preference would be reduced to 1 kcal mol^{-1}, because of the absence of an interaction with O(2) in A_2 and E_3.

For aglycones larger than methanol, and perhaps especially in polysaccharides, additional interactions can be expected to assume importance, and they may substantially modify these steric preferences, which, of course, arise solely from the fact that O(5) carries no ring substituents, while C(2) carries two.

It is important to form an accurate mental picture of what free-energy differences of this order would mean in terms of the population of rotamers. For the axial anomer, in which A_3 is virtually precluded, a steric preference of 1 kcal mol^{-1} for the A_1 rotamer would mean that, at 25°, *ca.* 15% of the A_2 rotamer would be present at equilibrium. When the preference is 2 kcal mol^{-1}, *ca.* 4% of A_2 would be present, and when it is 3 kcal mol^{-1}, *ca.* 1% of A_2 would be expected. For the equatorial anomer, the proportion of the less stable rotamers would, of course, be greater.

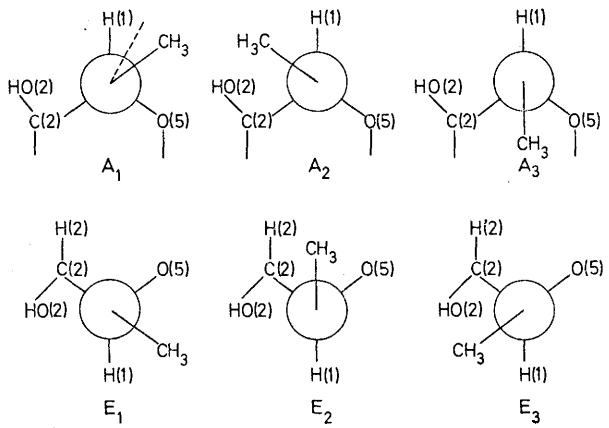

FIGURE 1 Rotamers generated about the C(1)–O(1) bond in methyl α-D-glucopyranoside (A_1—A_3) and in methyl β-D-glucopyranoside (E_1—E_3). The dotted line in A_1 shows the approximate position of the O(1)–C(4′) bond in the left-handed form of *V*-amylose

It is now necessary to consider how these populations could be modified by electronic effects.

exo-*Anomeric Effect.*[3,7]—Stoddart [7] has given a review of the *exo*-anomeric effect, and its relationship to the anomeric effect. Lemieux [8,9] had recognised that, for a given anomer, the dipole moments of the three rotamers would differ. That this should be so, can be seen in Figure 1, by considering the dihedral angle formed between the O(5)–C(1) bond, and the bond adjoining O(1) to the methyl carbon atom.

* It has been suggested [7] that *electronic effects* should be adopted as a general term for the anomeric and *exo*-anomeric effects, and related phenomena.

[4] H. S. Frank and W. Y. Wen, *Discuss. Faraday Soc.*, 1957, **24**, 133.

[5] E. L. Eliel, N. L. Allinger, S. J. Angyal, and G. A. Morrison, ' Conformational Analysis,' Interscience, New York, London and Sydney, 1965, pp. 356, 436.

[6] J. F. Stoddart, ' Stereochemistry of Carbohydrates,' Wiley–Interscience, New York, London, Sydney, and Toronto, 1971, p. 67.

[7] Ref. 6, pp. 72—87.

[8] R. U. Lemieux, Abstracts of papers, *Amer. Chem. Soc.*, 1959, **135**, 5E.

[9] R. U. Lemieux, in ' Molecular Rearrangements,' ed. P. De Mayo, Interscience, New York, London, and Sydney, 1964, vol. 2, p. 738.

However, it can best be seen from models, which show that, in the A_1 rotamer, the bisector of the bond angle between C(1), O(1), and the methyl carbon atom is exactly antiparallel to that of the bond angle between C(5), O(5), and C(1). On the other hand, in the E_3 rotamer, the corresponding bisectors are exactly parallel. *A priori* calculations with 2-methoxytetrahydropyran as a model indicate, in fact, that the net dipole moment should be zero in A_1, 2.60 D in E_3, and 1.84 D in the other rotamers.[3] This implies that, *in a non-polar solvent*, A_1 would be strongly preferred for the axial anomer (thus reinforcing the steric preference), while E_1 and E_2 would be preferred equally in the equatorial anomer.[3,10]

The *exo*-anomeric effect remained a theoretical idea until 1969, when Lemieux *et al.*,[3] after establishing conditions under which optical rotation can be validly applied to the study of rotameric populations,* made the remarkable observation that, at least for methyl 2-deoxypentopyranosides, *water has a strong capacity to stabilise the A_2 rotamer*. This implied that water is able not only to cancel the *exo*-anomeric effect, but also to overcome, at least in part, a steric barrier of *ca.* 1 kcal mol^{-1}.

Lemieux *et al.*[3] suggested that this phenomenon was due to the formation, by a single water molecule, of a hydrogen-bonded bridge between the ring and glycoside oxygen atoms, which would be possible in the A_2 rotamer, but not in A_1. We have tried with a model to build such a bridge, but it seems to entail much distortion of bond angles. Moreover, quantum mechanical calculations, which have been carried out in an attempt to understand the structure of liquid water, indicate that such a bridge, in which a water molecule is acting twice as a hydrogen donor, would be less stable than one in which it acts only once in that capacity.[11]

An alternative, and more general, postulate would be to regard the solvation of O(5) as tantamount to the introduction of substituents. Since the absence of such substituents in an aprotic solvent is the sole reason for the steric preference for the A_1 rotamer, it could then be readily understood why their introduction should be able to cancel it.

Amylose–Iodine Complex.—Reference is made to the notable series of papers by Rundle and his associates.[12–18] When an aliphatic alcohol such as butan-1-ol is added to an aqueous solution of amylose, a crystalline adduct separates.[19] Upon removal of solvent, the same crystalline form is retained, and the product is described as '*V*-amylose.' It consists of hexagonally close-packed helices of amylose, each containing six glucose residues per turn.[15] When this material is exposed to iodine vapour, it takes up one molecule of iodine (I_2) for every six glucose residues, without any substantial change in its crystalline form.† The iodine molecules are arranged in a row through the centre of the helices, in an end-to-end fashion.[14]

Systematic model building in the digital computer was carried out by Ramachandran and his co-workers,[20] in an attempt to arrive at a more detailed interpretation of the *X*-ray diffraction data of Rundle *et al.*[14,15] The data were more consistent with a left-handed than with a right-handed helical structure, and a recent re-investigation by French and Zaslow [21] has confirmed that the left-handed helix (Figure 2) is the correct one. The bond angles at the glycosidic linkages are not perfectly staggered in the helix; the O(1)–C(4′) bonds (*cf.* the O–CH_3 bond in Figure 1) make

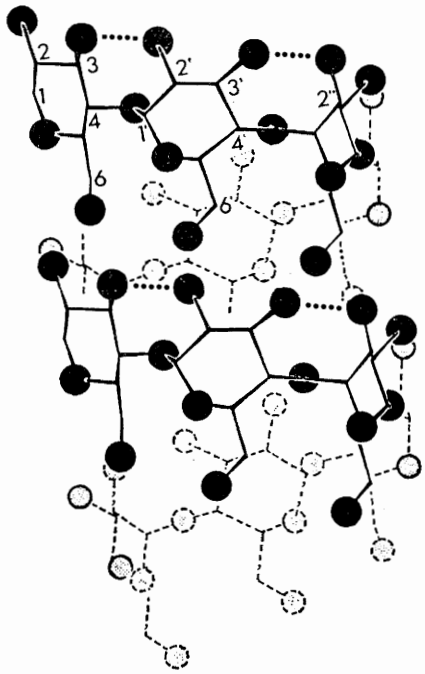

FIGURE 2 The left-handed *V*-helix of amylose (reproduced from ref. 49 by kind permission of Dr. B. Casu)

a dihedral angle of *ca.* 30° with the C(1)–H(1) bonds, and an angle of *ca.* 90° with the C(1)–O(5) bonds.[20,22] The rotamers can therefore be described as A_1, distorted significantly towards the A_2 form (see Figure 1).

The structure of the soluble, blue complex that is formed when a solution of iodine in aqueous potassium iodide is added to an aqueous solution of amylose is not quite so firmly based, but the demonstration of dichroism of flow,[12] the strongly co-operative nature of the binding mechanism,[17] and the existence of a well defined saturation limit [16] all

* These are essentially that there must be only one chiral centre at which rotamers can be generated, and that the molecule should be as rigid as possible whenever there is a possibility of solvent-induced changes in other pairwise interactions.

† In the presence of iodine or butan-1-ol, the helices are slightly more compact.[14,15]

[10] R. U. Lemieux and J. C. Martin, *Carbohydrate Res.*, 1970, **13**, 139.
[11] J. Del Bene and J. A. Pople, *J. Chem. Phys.*, 1970, **52**, 4858.
[12] R. E. Rundle and R. R. Baldwin, *J. Amer. Chem. Soc.*, 1943, **65**, 554.
[13] R. E. Rundle and D. French, *J. Amer. Chem. Soc.*, 1943, **65**, 558.
[14] R. E. Rundle and D. French, *J. Amer. Chem. Soc.*, 1943, **65**, 1707.
[15] R. E. Rundle and F. C. Edwards, *J. Amer. Chem. Soc.*, 1943, **65**, 2200.
[16] R. R. Baldwin, R. S. Bear, and R. E. Rundle, *J. Amer. Chem. Soc.*, 1944, **66**, 111.
[17] R. E. Rundle, J. F. Foster, and R. R. Baldwin, *J. Amer. Chem. Soc.*, 1944, **66**, 2116.
[18] R. S. Stein and R. E. Rundle, *J. Chem. Phys.*, 1948, **16**, 195.
[19] T. J. Schoch, *J. Amer. Chem. Soc.*, 1942, **64**, 2957.
[20] V. S. R. Rao, P. R. Sundararajan, C. Ramakrishnan, and G. N. Ramachandran, in 'Conformation of Biopolymers,' ed. G. N. Ramachandran, Academic Press, London, 1967, vol. 2.
[21] A. D. French and B. Zaslow, *J.C.S. Chem. Comm.*, 1972, 41.
[22] V. S. R. Rao and P. R. Sundararajan, in 'Solution Properties of Natural Polymers,' Special Publication No. 23, Chem. Soc., London, 1968, p. 173; V. S. R. Rao, N. Yathindra, and P. R. Sundararajan, *Biopolymers*, 1969, **8**, 325.

point to a linear arrangement of iodine atoms. Most definitive of all is the fact that extrapolation to zero concentration of iodide ions indicates a complex containing one iodine molecule for every six glucose residues, exactly as in the solid state.[16] In solution, of course, the helices must be more disordered than in the solid state.

Early doubts [12–18] about the state of the iodine in this soluble complex were resolved by the very precise physical measurements of Gilbert and Marriott,[23] which showed that the tri-iodine ion, I_3^-, is bound more strongly by amylose than molecular iodine, I_2, and that the selectivity for I_3^- increases with increasing degree of saturation. The results showed that, in the presence of an excess of potassium iodide, all saturated complexes would contain their iodine entirely as I_3^-, or, more correctly, as a polymer of this ion, $(I_3^-)_n$.

The polymeric ion is probably stabilised by the resonance of unlocalised electrons.[23] This idea accounts convincingly for the observed co-operativity [17,23] and the shift in the absorption spectrum towards longer wavelengths as the chain length of the amylose increases.[16] Gilbert and Marriott considered it unlikely that potassium ions would enter the helix, and proposed that they would be present, in solution, as counterions.[23] Since the complex is a kind of polyelectrolyte, it was pointed out that any increase in ionic strength should stabilise it.[23]

These results are confirmed and supplemented by work on the Schardinger α-dextrin (cyclohexa-amylose),[24] which is a good model for amylose, since it represents a single turn of the helix. The formation constants for reactions (1)—(4) are reported by Duke [25] for an unstated temperature.

$$\alpha + I_2 \rightleftharpoons \alpha,I_2;\ K = 2.0 \times 10^3 \qquad (1)$$

$$\alpha,I_2 + I^- \rightleftharpoons \alpha,I_3^-;\ K = 1.35 \times 10^5 \qquad (2)$$

$$\alpha,I^- + I_2 \rightleftharpoons \alpha,I_3^-;\ K = 2.0 \times 10^7 \qquad (3)$$

$$\alpha + I^- \rightleftharpoons \alpha,I^-;\ K = 13.5 \qquad (4)$$

The capacity of the α-dextrin to bind iodide ions alone was confirmed by free-boundary electrophoresis,[26] and the isolation of a crystalline complex, α,KI.[25]

The significance of these formation constants for the present work lies, of course, in their *relative* magnitudes. Their absolute values must be higher than those for amylose, because, in the α-dextrin, the glucose residues are already firmly held in the correct conformation for adduct formation. On the other hand, it must be remembered that the binding of I_3^- in amylose is co-operative,[17] whereas in the isolated α-dextrin molecules it cannot be. It is scarcely possible for the binding of I^- alone to be co-operative, while the situation regarding molecular iodine is uncertain.*

One more piece of information is needed to give a fairly clear picture of the likely composition of an aqueous solution containing amylose in the presence of an excess of iodine and potassium iodide. This is the formation constant for I_3^- [reaction (5)]. It may be calculated [23] from the data of Jones and Kaplan at 20°.[27]

$$I_2 + I^- \rightleftharpoons I_3^-;\ K = 8.07 \times 10^2 \qquad (5)$$

It is evident that it should be a simple matter to find conditions under which the only significant species in solution are (a) amylose containing I_3^-, (b) unbound I_3^-, (c) excess of I^-, and (d) potassium ions. The amount of I_3^- in the complex (a) can be measured spectrophotometrically,[16,28,29] and we propose to use this as a measure of the ease with which starch and soluble derivatives of amylose will take up the helical, *V*-conformation in the presence of different acids and salts. The use of the spectrophotometric method to measure the same property is, of course, well precedented in its application to characterise the degree of branching of amylopectins and glycogens,[16,28–31] and it has also been used to study the effect of pH upon the capacity of carboxymethylamylose to assume the *V*-conformation.[32,33]

Previous Work on the Effect of Salts upon Complex Formation.—The ability of inorganic ions to change the iodine-binding capacity of starch fractions has been long recognised. The first well authenticated report is probably that of Bates *et al.*,[34] who noted that the amount of iodine that is firmly bound by amylose decreases as the concentration of potassium iodide in the solution increases. This was shown by potentiometric titration of a solution of amylose in aqueous potassium iodide with a solution of iodine in potassium iodide of the same concentration. The end-point of the titration was taken as the point at which the activity of iodine in the solution rapidly increases.[34] This analytical method is now used routinely in the field,[35] and the dependence of the end-point upon the concentration of potassium iodide is cited as calling for carefully standardised conditions in its application.[36]

These findings were confirmed by Baldwin *et al.*,[16] who used spectrophotometric titration as an independent method for measuring the uptake of iodine. In this method, the conditions of titration are similar, but the uptake of iodine is measured as an increase in the absorbance at a wavelength near to the rather broad absorption maximum (600—680 nm). When the absorbance was plotted against the amount of added iodine, a series of curves was obtained which were different in the presence of different concentrations of potassium iodide. In each case, there was an initial, rapid increase in absorbance, followed by a very slow, further increase. The two linear parts of each curve were extrapolated, and the end-point was taken as the amount of added iodine corresponding to the point at which they intersect.[16]

For concentrations of potassium iodide up to 0.5M, the

*A mechanism has been proposed [18] for the co-operative binding of I_2, but it was based upon the belief that I_2 is bound as such in the presence of KI. Co-operativity has been experimentally demonstrated only in systems that contain KI.[17]

[23] G. A. Gilbert and J. V. R. Marriott, *Trans. Faraday Soc.*, 1948, **44**, 84.
[24] D. French, *Adv. Carbohydrate Chem.*, 1957, **12**, 249.
[25] H. A. Duke, Ph.D. Thesis, Iowa State College, 1947.
[26] E. Norberg and D. French, *J. Amer. Chem. Soc.*, 1950, **72**, 1202.
[27] G. Jones and B. B. Kaplan, *J. Amer. Chem. Soc.*, 1928, **50**, 1845.
[28] R. McCready and W. Z. Hassid, *J. Amer. Chem. Soc.*, 1943, **65**, 1154.
[29] G. A. Gilbert and S. P. Spragg, *Methods Carbohydrate Chem.*, 1964, **4**, 168.
[30] J. Hollo and J. Szejtli, *Die Stärke*, 1958, **10**, 49.
[31] S. Ono, S. Tsuchibashi, and T. Kuge, *J. Amer. Chem. Soc.*, 1953, **75**, 3601.
[32] V. S. R. Rao and J. F. Foster, *Biopolymers*, 1963, **1**, 527.
[33] V. S. R. Rao and J. F. Foster, *Biopolymers*, 1965, **3**, 185.
[34] F. Bates, D. French, and R. E. Rundle, *J. Amer. Chem. Soc.*, 1943, **65**, 142.
[35] T. J. Schoch, *Methods Carbohydrate Chem.*, 1964, **4**, 157.
[36] C. T. Greenwood, *Adv. Carbohydrate Chem.*, 1956, **11**, 367.

end-points gave the empirical relationship (6) where $[I_2]$ is expressed as moles of iodine per glucose residue, and

$$1/[I_2] = 6 + 2.4[KI]^{0.25} \quad (6)$$

[KI] is the molarity of potassium iodide. Since the absorbance is approximately * proportional to the amount of bound iodine, the terminal parts of each curve gave a series of nearly parallel straight lines of very low slope,[16] which are referred to in this paper as ' plateaux.'

Baldwin *et al.*[16] attempted to explain these phenomena by proposing that I_3^- was competitively displacing I_2 from the helix. This suggestion is plausible because, under the conditions of the titrations, the ratio of I_3^- to I_2 in the system would have been constant. However, in the light of later work,[23,25] it is difficult to see how it can be correct. Even with the Schardinger α-dextrin, for which there are no co-operative effects, the selectivity for I_3^- is so high that, in the lowest concentration of potassium iodide used by Baldwin *et al.* (10^{-3}M), the ratio of I_3^- to I_2 in the complex would have been 135 : 1 [equation (2)]. The other concentrations used were 0.01, 0.05, and 0.50M. With amylose itself, the co-operative effect would be expected [23] to increase this selectivity, and indeed, for a concentration of potassium iodide of 0.02M, Gilbert and Marriott [23] found by direct analysis that all the iodine was bound as I_3^-.

It thus appears that relationship (6) noted by Baldwin *et al.*[16] must describe some destabilising effect of potassium iodide upon the amylose–I_3^- complex, which is still unexplained. The unusual form of this relationship is noteworthy. It is not possible to explain it with any simple model that assumes the competitive or non-competitive binding of iodide or potassium ions. The low value of the exponent (0.25) suggests that it would be nearer the truth to assume that the phenomenon is related in some way to the *mean distance* between the amylose molecules and the ions (in which case, of course, it would be 0.33).

The need for a more general hypothesis to explain these phenomena is confirmed by the fact that other salts also change the capacity of starch fractions to bind iodine. For example, ammonium sulphate [37] and calcium chloride [38] both increase the iodine-binding capacity, and the addition of these salts is used routinely, as a practical device for increasing sensitivity, when the method is used to characterise branched fractions [39,40] and fractions of low molecular weight.[40] Implicit in all these applications is the clear recognition that it is impossible to achieve a similar increase in the uptake of iodine, merely by increasing the concentration of iodine in the system.

Schlamowitz, in his paper about glycogen,[39] came nearest to proposing a general hypothesis when he suggested that ammonium sulphate stabilised the helix because its interior was relatively hydrophobic, like that of a globular protein. He also recognised that it is impossible to explain any of these phenomena on the assumption that the materials contain only a single kind of binding-site. In the light of the numerical data that have been cited [equations (1)—(5), and ref. 23] it is obvious that any given fraction of starch should always give the same saturation limit, if it contained a fixed proportion of identical binding sites. The simplest assumption that seems to fit the facts is that there are two different kinds of binding site, one of which binds iodine very strongly, and the other very weakly, and that the relative proportion of these changes in the presence of different salts.

Schlamowitz also recognised the physical basis for this idea.[39] He pointed out that glycogens must contain a wide spectrum of different binding sites, by virtue of the fact that they contain a distribution of linear sequences of different lengths. Because of the strongly co-operative binding mechanism, the longer sequences are filled first.[17] As the linear segments get shorter, this source of stability diminishes, and at the same time, steric effects should start to impair the ability of each glucose residue to take up the correct geometry for helix formation. Schlamowitz considered that a proportion of the shorter sequences would always be empty, and that ammonium sulphate increases the number of sequences that are able to take up iodine.[39]

These ideas are not invalidated by the behaviour of amylose,[16,34,36] because it is difficult to obtain amylose completely free from branched material,[16] and even if this were accomplished, the material would still contain a distribution of chains of different lengths. Moreover, Baldwin *et al.* suggested, and obtained indicative evidence for, the idea that iodine is bound more weakly near the ends of the chains than at the centre.[16] The fact that the plateaux [16] were not perfectly horizontal proves that there was more than one kind of binding site, and supports the idea that potassium iodide changed the relative proportion of these.

The present work appears to be the first systematic study of the effect of salts on the complex, but it is restricted to the salts of monovalent, closed-shell cations, to avoid difficulties in interpretation arising from the possible formation of co-ordination complexes.

Plan of Experiment.—We are not interested in studying the effect of inorganic ions upon the activity coefficient of I_3^-, or the competitive binding of species other than I_3^-. It is only possible to avoid these difficulties by carrying out the experiments in the presence of a massive excess of I_3^-, and measuring the differences between the heights of the plateaux.

This plan of experiment has an evident parallel in enzymology,[41] the heights of the plateaux corresponding to the maximum velocity, $V_{max.}$, of an enzymic reaction. Competitive inhibition cannot change $V_{max.}$, but only the concentration of substrate that is required to give it, so that it should be possible to avoid errors arising from this by titrating each sample until the plateau condition is reached. The phenomena that we propose to study bear a certain resemblance to non-competitive inhibition and activation, which do change $V_{max.}$, but we do not expect it to take place by direct site-binding.†

It was evident that pure amylose of high molecular weight would be an unsuitable material for study, because

* The absorbance would be strictly proportional to the amount of bound iodine only when the amylose molecules are all of the same length. This is because the longer chains are filled first,[17] and the shorter ones give a different absorption spectrum.[16]

† Interestingly, the quantitative treatment of non-competitive inhibition [41] predicts a relationship that is identical in form to (6), except that the exponent in the concentration of inhibitor is, of course, unity.

[37] J. B. Sumner and G. F. Somers, *Arch. Biochem.*, 1944, **4**, 7.
[38] W. L. Deatherage, M. M. MacMasters, and C. E. Rist, *Trans. Amer. Assoc. Cereal Chemists*, 1955, **13**, 31.
[39] M. Schlamowitz, *J. Biol. Chem.*, 1951, **190**, 519.
[40] C. R. Colburn and T. J. Schoch, *Methods Carbohydrate Chem.*, 1964, **4**, 161.
[41] M. Dixon and E. C. Webb, ' Enzymes,' Academic Press, New York, 1958.

it would provide mainly only one kind of binding site.* There was also the practical consideration that its solutions are unstable in water, because of retrogradation, and it salts out very easily. Soluble starch, consisting mainly of degraded amylopectin, was therefore chosen for most experiments. Other experiments were carried out with derivatives of amylose, which were expected to contain different binding sites because of the expected lack of uniformity in the distribution of substituents along the chains.

The optical density was measured at 680 nm, because of the evidence [16] that this is a specific measure of I_3^-, polymerised in long sequences inside the *V*-helix. It is perhaps possible that I_3^- can be bound to the glucans in other ways, but it is very unlikely that such binding would contribute to the absorbance at 680 nm.

In working with bromides, special attention was given to the possibility of the competitive binding of I_2Br^-, and to the effect that the formation of this species would have upon the activity of I_3^-. The formation constant of

$$I_2 + Br^- = I_2Br^-; \; K = 10.5 \qquad (7)$$

I_2Br^- at 25° is reported [42] in equation (7). The corresponding figure for I_2Cl^- at 25° is given [43] in equation (8).

$$I_2 + Cl^- = I_2Cl^-; \; K = 1.66 \qquad (8)$$

The existence of I_2F^- in aqueous solutions does not appear to have been reported.

Criteria of Mechanism.—In Parts I [2] and II,[1] the influence of inorganic ions upon the magnitude of the anomeric effect was studied by measuring the ratio (K_β/K_α) of the rates of hydrolysis of anomeric pairs of D-glucopyranosides in different mineral acids. In this way, all medium effects upon the rest of the molecules were automatically compensated for, and a specific measure was obtained of the effect of the ions upon the capacity of water to solvate the ring and glycoside oxygen atoms.

In the present work, we abandon the theoretical safety provided by this kind of experiment, and try to interpret medium effects upon a single kind of molecule. We therefore have to consider other possible explanations for the effects observed, and try to establish criteria for distinguishing between them. We have considered just two alternatives, both borrowed from the field of protein chemistry.[44]

The first is that of Schlamowitz,[39] who points out that the inside of the *V*-helix is a non-polar environment, compared at least to that of water. The ability of an inorganic salt to stabilise the so-called ' hydrophobic interactions ' in globular proteins is recognised [44] to be closely correlated with its ability to salt-out non-polar compounds from aqueous solution.[45,46] This ability is measured by a salting-out parameter, k_s, which is defined by the Setchénow equation (9) [47] where f is the activity coefficient of the non-polar solute, and C_s is the molarity of salt. To a first approximation,[45] the value of k_s is simply the sum of the separate contributions of the anion and cation, and these contributions have led to the recognition of two well defined salting-out orders, which seem to hold true for all non-polar compounds [sequences (10) and (11)].[45,46]

$$\log f = k_s C_s \qquad (9)$$

$$SO_4^{2-} > OH^- > F^- > Cl^- > Br^- > I^- \qquad (10)$$

$$Na^+ > K^+ > Li^+ = Rb^+ > Cs^+ \qquad (11)$$

Special attention is called to the order for alkali-metal ions (11), because it leads to predictions quite different from those of the water-orientation hypothesis.[1] Thus, the latter hypothesis predicts that the capacity of any given anion to increase the magnitude of the anomeric effect should diminish as the ionic radius of the associated cation decreases. If, therefore, the effect of a salt upon the iodine-binding capacity were due solely to its influence upon the magnitude of the anomeric effect, then, for any given anion, the limiting uptake of iodine should decrease as the cation is varied in the order (12).

$$Cs^+ > Rb^+ > K^+ > Na^+ > Li^+ \qquad (12)$$

The salting-out order for anions (10) is not so useful, because it leads to similar predictions in both cases. However, it is important to notice that all the anions in (10), as well as all the cations in (11), *invariably salt-out non-polar compounds.*[45] The only purely inorganic compound that has been reported to salt-in a non-polar solute seems to be perchloric acid.[45] Von Hippel and Schleich [44] have previously called attention to the diagnostic value of this fact, and we have also applied it in Part II.[1] It is evident that the hypothesis of Schlamowitz [39] explains the behaviour of ammonium sulphate,[37,39] but not that of potassium iodide.[16,34]

The second possibility that we have considered is that inorganic ions may modify the stabilities of any intramolecular hydrogen bonds in amylose. An *X*-ray analysis of the potassium acetate complex of the Schardinger α-dextrin led Hybl *et al.*[48] to conclude that hydrogen bonding between O(2) of one glucose residue and O(3) of the next in the ring was likely in the crystalline state. Casu *et al.*[49] have obtained indicative evidence for weak hydrogen-bonding of the same kind in solutions of amylose in dimethyl sulphoxide, and the suggested bonds are shown in Figure 2.

In general, one would expect this kind of hydrogen bonding to become stronger as competitive hydrogen bonding with the solvent molecules decreases. We have pointed out that the extent to which the hydroxy-groups are hydrated in solution is not necessarily the same as the extent to which the ring and glycoside oxygen atoms are hydrated.[1] This is because the former can be hydrated both as proton acceptors and donors, whereas the latter can be hydrated as proton acceptors only.[1] Thus, a salt or acid that increases the proton-accepting capacity of water at the expense of its proton-donating capacity may be a good

*A referee has pointed out that this would not necessarily be true, if the system were not in rapid thermodynamic equilibrium.

[42] R. Guidelli and F. Pergola, *J. Inorg. Nuclear Chem.*, 1969, **31**, 1373.

[43] A. I. Popov, in ' Halogen Chemistry,' ed. V. Gutmann, Academic Press, New York and London, 1967, p. 225.

[44] P. H. von Hippel and T. Schleich, in ' Structure and Stability of Biological Macromolecules,' eds. S. N. Timasheff and G. D. Fasman, Biological Macromolecules Series, Marcel Dekker, New York, 1969, vol. 2, p. 417.

[45] W. F. McDevit and F. A. Long, *J. Amer. Chem. Soc.*, 1952, **74**, 1773.

[46] F. A. Long and W. F. McDevit, *Chem. Rev.*, 1952, **51**, 119.

[47] M. Setchénow, *Ann. chim. phys.*, 1892, **25**, 226.

[48] A. Hybl, R. E. Rundle, and D. E. Williams, *J. Amer. Chem. Soc.*; 1965, **87**, 2779.

[49] B. Casu, M. Reggiani, G. G. Gallo, and A. Vigevani, in ' Solution Properties of Natural Polymers,' Special Publication No. 23, Chemical Society, London, 1968, p. 217.

solvent for a carbohydrate, while at the same time it selectively dehydrates the ring and glycoside oxygen atoms, leading to an increase in the magnitude of the anomeric effect.

Likely examples of this can be seen in the fact that sulphuric and phosphoric acids both increase the magnitude of the anomeric effect, but are nevertheless good solvents for the methyl glucopyranosides.[2] Relatively concentrated solutions of these two acids (60% w/w sulphuric acid or 80% w/w phosphoric acid) are powerful solvents for cellulose [50] and amylose,[51] and it is interesting that solutions of amylose in these two acids stain intensely with iodine until extensive hydrolysis has occurred.[51]

These facts suggest a possible experimental basis for distinguishing between a phenomenon that is primarily a function in the extent of intramolecular hydrogen bonding, and one which is mainly a function in the magnitude of the anomeric effect. Thus, in the former case, one would expect a correlation with the hydration of the molecules as a whole, while in the latter, no correlation should exist.

As a general method for studying the ability of salts and acids to enhance or diminish hydration of the starch molecules as a whole, the simple device was adopted of cross-linking starch with epichlorohydrin, and measuring the changes in volume that occurred when the resultant gel was immersed in the solutions. It should perhaps be noted that the cross-linking introduces ether systems into the material, so that the results may be spuriously biased in favour of a positive correlation with the anomeric effect (*cf.* a similar correlation in methyl cellulose [1]).

EXPERIMENTAL

Materials.—*V*-Amylose, having a blue-value [28,29,52] of 1.28, was supplied by Avebe AG. Soluble starch was supplied by Riedel de Haën AG. It had a blue-value [52] of 0.136, and periodate oxidation [52] indicated the presence of one end-group for every 17 glucose residues. Diethylaminoethylamylose hydrochloride, having a degree of substitution (d.s.) of 0.29, was prepared and characterised as described previously.[53] Amylose sulphate, having d.s. 0.35, was prepared as described by Whistler,[54] and isolated as its sodium salt. Carboxymethylamylose (Na^+ salt), having d.s. 0.26, was prepared as described by Green [55] for the corresponding cellulose derivative. Polyvinyl alcohol, described as 'Elvanol Grade 52-22,' was supplied by DuPont. The other reagents were of Merck analytical quality.

Spectrophotometric Assays.—Absorption spectra were obtained with a Coleman–Hitachi model 124 spectrophotometer, coupled with a Hitachi–Perkin-Elmer model 165 recorder. Routine measurement of optical densities was made with a Hitachi–Perkin-Elmer model 139 spectrophotometer. The path length was 1 cm throughout, and the temperature was 20°.

The procedure was essentially the same as that used for characterising starch fractions.[28,29] The standard iodine solution, which was diluted as required, consisted of iodine (4.00 g) and potassium or sodium iodide (40 g) in water (500 ml). The stock solutions of the various polymers were freshly prepared each day by heating at 80° in distilled water, and consisted of the following concentrations (w/v): soluble starch, 0.1%; diethylaminoethylamylose hydrochloride, 1.0%; amylose sulphate, 5.0%; carboxymethylamylose, 0.1%; and polyvinyl alcohol, 1.0%.

For each assay, a portion (1 ml) of polymer solution was first mixed well with 20 ml of the solution of salt or acid. Iodine solution (0.5 ml) was then added, and, after mixing, the solution was kept 10 min at 20° before reading the optical density at 680 nm (for starch derivatives) or 500 nm (for polyvinyl alcohol), against blanks containing iodine and the salt or acid alone. In working with relatively concentrated acids (>2M), the acid was first brought to 20°, and the optical densities were read immediately after mixing.

Cross-linking of Starch with Epichlorohydrin.—A solution (5% w/v) of starch in aqueous sodium hydroxide (20% w/v; 250 ml) was shaken vigorously with epichlorohydrin (50 ml) until it started to exhibit non-Newtonian ('long') flow (*ca.* 10 min). It was then poured into a shallow rectangular tray (30 × 20 cm). The surface was covered with a thin layer of epichlorohydrin, and the tray was covered with glass to prevent evaporation. After 24 h, the gel (*ca.* 3 mm thick) was cut into strips (10 × 1 cm), and washed exhaustively with distilled water. The strips were then transferred to Petri dishes containing 50 ml of salt or acid, and any change in length after 24 h was measured with a Vernier gauge. The measurements were made in triplicate, and close agreement (±1%) between parallels was noted. The changes in length were reversible in all instances reported. Confirmation that the gel was freely permeable to the ions was obtained by titrimetry (of acids) or gravimetry (of salts) leached out by water after equilibration.

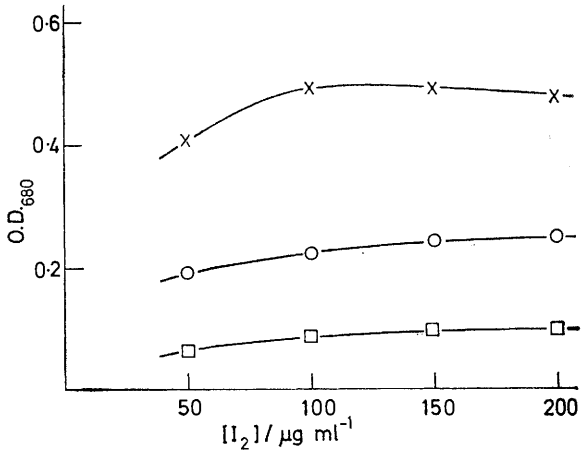

FIGURE 3 Effect of total concentration of iodine (calculated as I_2) upon the optical density at 680 nm of solutions (50 μg ml^{-1}) of starch in 4M-potassium fluoride (×), water (○), and 4M potassium iodide (□) at 20°

RESULTS

Evidence for a Salt-induced Change in the Number of Binding Sites.—For given concentrations of starch or substituted amylose, increasing concentrations of iodine–iodide mixture led, in every salt or acid, to fairly well defined plateaux or maxima in the optical density at 680 nm. The results shown in Figure 3 for solutions of

[50] G. Jayme and F. Lang, *Methods Carbohydrate Chem.*, 1963, **3**, 75.
[51] T. J. Painter, unpublished observation.
[52] M. Fahmy Ishak and T. J. Painter, *Carbohydrate Res.*, 1974, **32**, 227.
[54] R. L. Whistler, *Methods Carbohydrate Chem.*, 1972, **6**, 426.
[55] J. W. Green, *Methods Carbohydrate Chem.*, 1963, **3**, 322.

starch in water, 4M-potassium iodide, and 4M-potassium fluoride were typical.

When the optical density passed through a maximum, as shown for potassium fluoride, it was usually with a salt that increased the absorbance, and it was attributed to the excess of potassium iodide that was unavoidably present in the added solution of iodine.

The absorption spectra in the same three media are shown in Figure 4. The curves differ not only with respect to the positions of the maxima, but also in shape. In any given medium, the absorption spectrum was virtually independent of the amount of added iodine. This is illustrated, for solutions of starch in water, in Figure 5.

Similar experiments were carried out on polyvinyl alcohol, because this polymer also binds iodine,[56,57] but contains no acetal systems, and cannot therefore exhibit an anomeric effect. A practical problem was that this polymer continued to bind iodine until it precipitated from solution

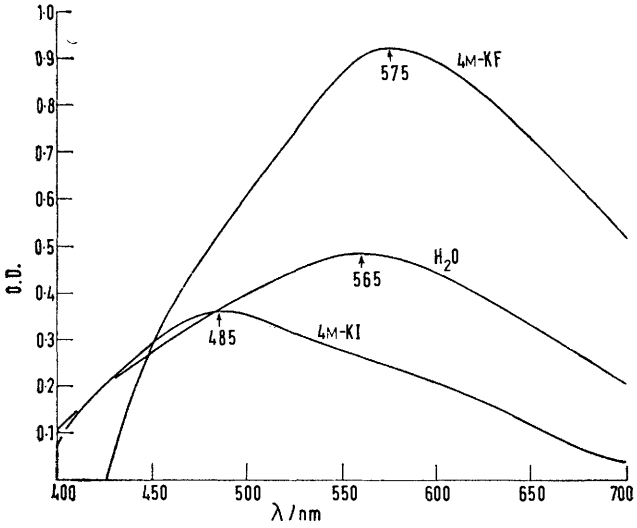

FIGURE 4 Absorption spectra of solutions of starch (50 μg ml^{-1}) and iodine (200 μg ml^{-1}) in 4M-potassium fluoride, water, and 4M-potassium iodide at 20°

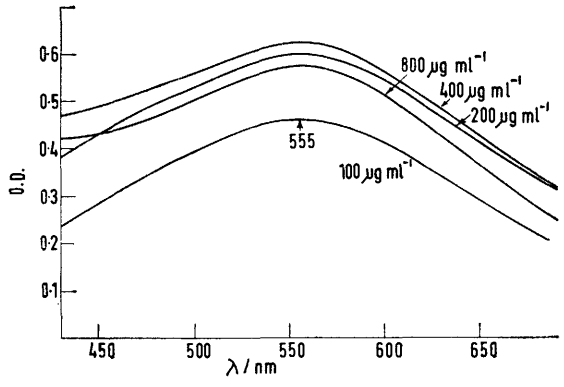

FIGURE 5 Absorption spectra of aqueous solutions of starch (50 μg ml^{-1}) containing 100, 200, 400, and 800 μg ml^{-1} of total iodine at 20°

as a purple gum. The data in Figure 6 represent measurements that were made up until this happened in water, 4M-potassium iodide, and 0.5M-potassium fluoride. The polyvinyl alcohol was salted-out by higher concentrations of potassium fluoride.

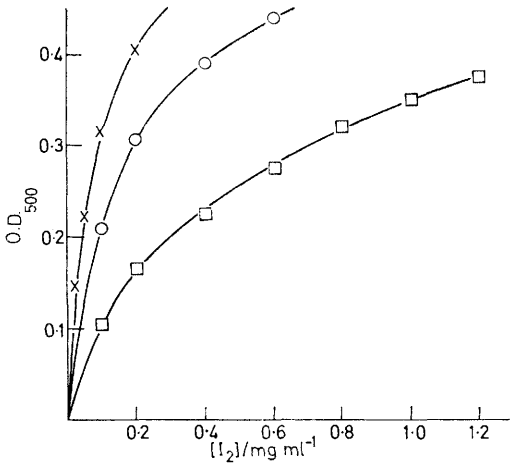

FIGURE 6 Optical density at 500 nm and 20° of polyvinyl alcohol (0.5 mg ml^{-1}) in 0.5M-potassium fluoride (×), water (○), and 4M-potassium iodide (□), as a function of the total concentration of added iodine (calculated as I_2)

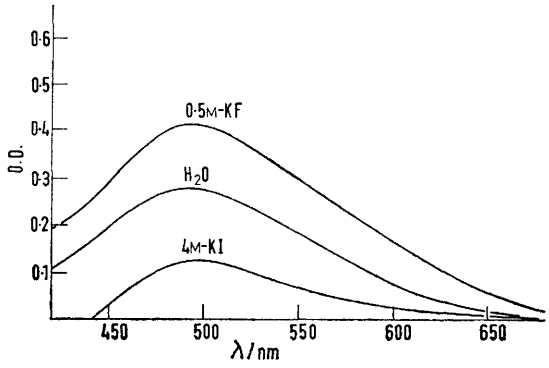

FIGURE 7 Absorption spectra of solutions of polyvinyl alcohol (0.5 mg ml^{-1}) and iodine (0.2 mg ml^{-1}) in 0.5M-potassium fluoride, water, and 4M-potassium iodide at 20°

With this system, it was clearly impossible to ascertain by direct measurement whether different maxima would be obtained, but it was possible to measure the ratios of the abscissae for given ordinates, and the results are given in Table 1. The near constancy in the ratios gives no

TABLE 1

Ratios of the concentrations (C_{KF}, C_{H_2O}, and C_{KI}) of total iodine required to produce given optical densities at 500 nm in solutions (500 μg ml^{-1}) of polyvinyl alcohol in 0.5M-potassium fluoride, water, and 4M-potassium iodide

Optical density	C_{KF}/C_{H_2O}	C_{H_2O}/C_{KI}
0.10	0.40	0.36
0.20	0.45	0.30
0.25	0.45	0.28
0.30	0.45	0.28
0.35	0.44	0.29
0.40	0.45	

[56] D. A. Godina and G. P. Faerman, *Zhur. obshchei Khim.*, 1967, **37**, 597.

[57] K. Bolewski and A. Buraczewska, *Acta Polonica Pharm.*, 1970, **27**, 33.

indication of the development of different maxima. The absorption spectra in the same three media are shown in Figure 7, and are seen to be almost identical.*

Choice of Conditions for Measurement of Limiting Optical Densities.—Experiments similar to that shown in Figure 3 were carried out with the highest concentration of every salt or acid that it was desired to study, and although the positions of the maxima were not always quite the same, it was decided to choose a fixed iodine concentration of 200 μg ml^{-1} for the remainder of the work. The error incurred in this way was not more than ±5%. In the case of starch, this concentration corresponded to a 16-fold molar excess of I_3^-, calculated on the assumption of a maximum binding capacity of 1 mol of I_3^- per six glucose residues. However, the blue-value of the starch in water was only about one-tenth of that of amylose, so that the true molar excess in water was close to 160-fold. The concentrations of the amylose derivatives were adjusted

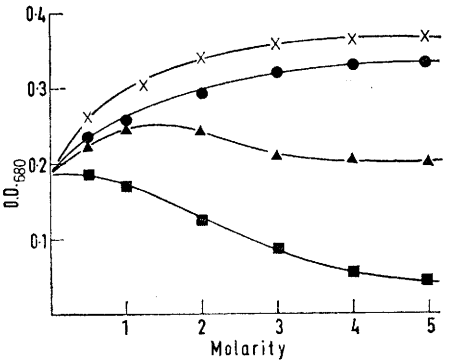

FIGURE 8 Effect of sulphuric (×), phosphoric (●), hydrochloric (▲), and hydrobromic (■) acids upon the limiting optical density of starch at 680 nm and 20°

to give optical densities in the same range of values, so that the molar excess of iodine in these cases should have been of the same general order.

In working with concentrated solutions of bromides, it was found that higher concentrations of iodine were not needed to bring the optical density within the plateau region, in spite of the expected formation of I_2Br^- [equation (7)]. This was explained by the discovery that it made little difference to the optical density, whether the iodine was added as a solution in potassium iodide or potassium bromide. It thus appeared that starch, when it is saturated with I_2Br^-, gives almost the same optical density at 680 nm as it does when it is saturated with I_3^-.

Effect of Mineral Acids upon the Iodine-binding Capacity of Starch and Diethylaminoethylamylose.—Figure 8 shows the effect, upon the limiting optical density of starch, of the four acids that were investigated in Parts I [2] and II,[1] and shown to change the magnitude of the anomeric effect to different extents. The concentrations of the acids are expressed as molarities, but the large differences between the curves do not disappear if the acid concentrations are expressed as ionic strength or as the Hammett function (H_0).

Whereas the earlier experiments on the anomeric effect [1,2] were carried out at 40—70°, it was unfortunately necessary to carry out the present ones at 20°. This was because the starch would have been hydrolysed too quickly at 40—70°, and the spectrophotometer was not designed to

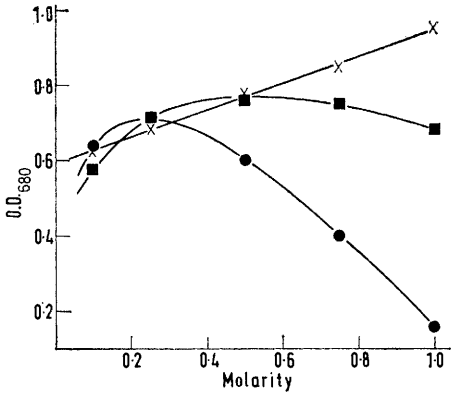

FIGURE 9 Effect of sulphuric (×), hydrochloric (■), and hydrobromic (●) acids upon the limiting optical density of diethylaminoethylamylose at 680 nm and 20°

operate at temperatures above 40°. However, it is at least clear that, in a relative sense, there is a positive correlation between the effect of the acids upon the anomeric effect, and their effect upon the limiting optical density.

Diethylaminoethylamylose, having a degree of substitution of 0.29, was examined because, even when saturated with I_3^-, it would still carry a net positive charge, and would therefore be expected to show a greater sensitivity to differences in the anion of the acid than ordinary starch. The results (Figure 9) have to be interpreted as a general effect of ionic strength upon the mutual repulsion of the cationic substituents, upon which is superimposed some specific effect of the different anions.

Effect of Alkali-metal Halides upon Starch.—The results for all the halides of sodium, potassium, and caesium are shown in Figures 10—12, respectively. The relatively low solubility of sodium fluoride prevented the study of concentrations higher than 0.5M. Lithium fluoride could not

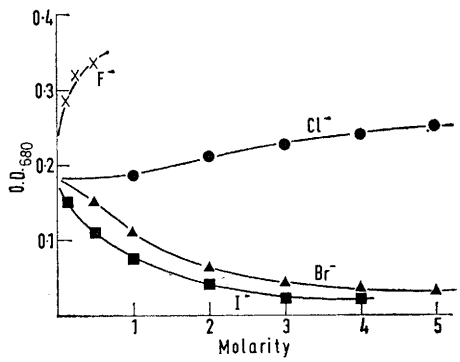

FIGURE 10 Effect of the halides of sodium upon the limiting optical density of starch at 680 nm and 20°

be studied because of its very low solubility, but the results for the chloride, bromide, and iodide are shown in Figure 13.

It was concluded that, for chlorides, bromides, and iodides, the limiting optical density decreased as the cation was varied in the order: $Cs^+ > K^+ > Na^+ > Li^+$, a result

* They are not identical at wavelengths below 450 nm, but in this region the iodine itself is absorbing strongly, allowing very little light through the cell. The apparent differences are therefore probably spurious. Bizarre effects of the same kind can be seen in Figure 5.

that maybe compared with series (12). For fluorides, however, the corresponding order was $Na^+ > K^+ > Cs^+$, which agrees with series (11).

Effect of Alkali-metal Halides upon Amylose Sulphate and Carboxymethylamylose.—It is evident from Figures 10—13 that differences in the identity of the anion affect the iodine-binding capacity much more than differences in the cation. These latter differences are, however, important

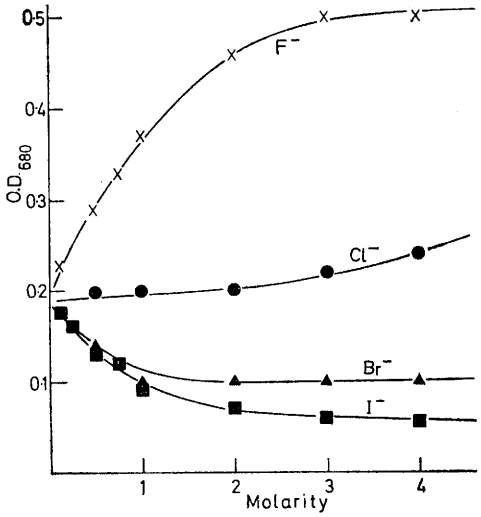

FIGURE 11 Effect of the halides of potassium upon the limiting optical density of starch at 680 nm and 20°

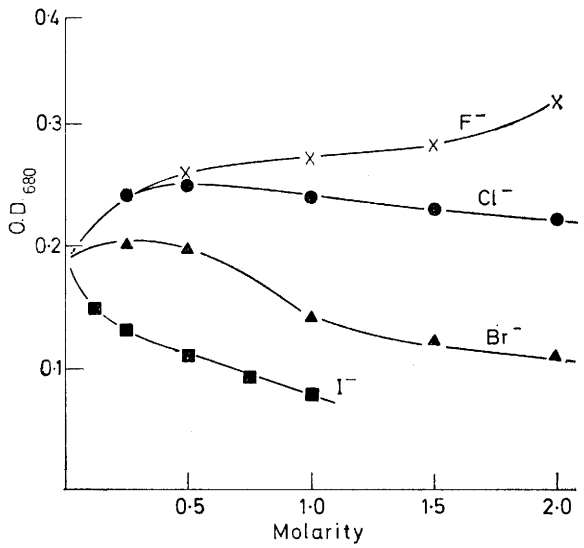

FIGURE 12 Effect of the halides of caesium upon the limiting optical density of starch at 680 nm and 20°

as a criterion of mechanism. Anionic derivatives of amylose were therefore studied, in an attempt to increase the sensitivity to cations, and, hence, to demonstrate the differences between them more clearly.

The behaviour of amylose sulphate with the chlorides, bromides, and iodides of lithium, sodium, and potassium is shown in Figure 14. Unfortunately, the fluorides of sodium and potassium caused precipitation of the complex, even at low concentrations, and it is only possible to report that they both caused a sharp increase in iodine-binding capacity.

It should be noted that, for these solutions of 1 : 1 electrolytes, the ionic strength is the same as the molarity. The

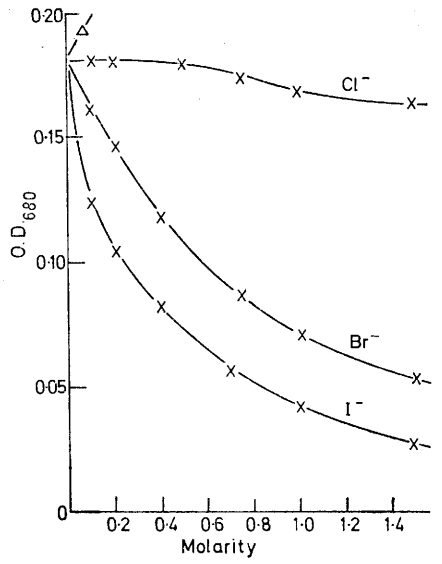

FIGURE 13 Effect of the halides of lithium upon the limiting optical density of starch at 680 nm and 20°. Because of its very low solubility, only one point (△) is shown for the fluoride

results again have to be interpreted as a general, stabilising effect of ionic strength upon the complex, superimposed

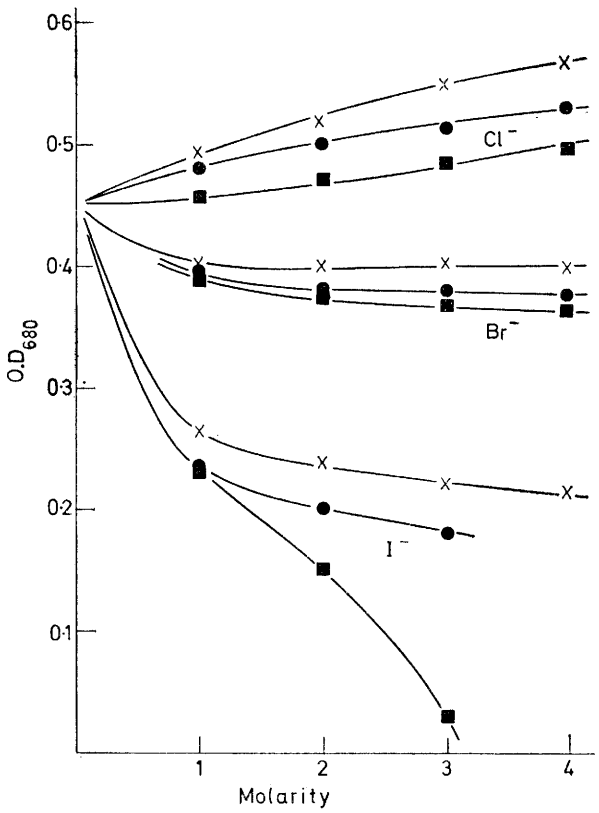

FIGURE 14 Effect of the chlorides, bromides, and iodides of sodium (●), lithium (■), and potassium (×) upon the limiting optical density of amylose sulphate at 680 nm and 20°

upon an evidently more important effect that is dependent upon the identities of the individual anions and cations.

The behaviour of carboxymethylamylose in the chlorides of lithium, sodium, and potassium is shown in Figure 15, and it is once again in agreement with series (12).

Effect of Other Cosolutes upon Starch.—A selection of data of general interest is given in Figure 16. The possibilities for making comparisons of interest were limited by the low solubilities of many salts. In the possible range of comparison (up to 0.4M), there was no significant difference in the behaviour of sodium sulphate and potassium sulphate.

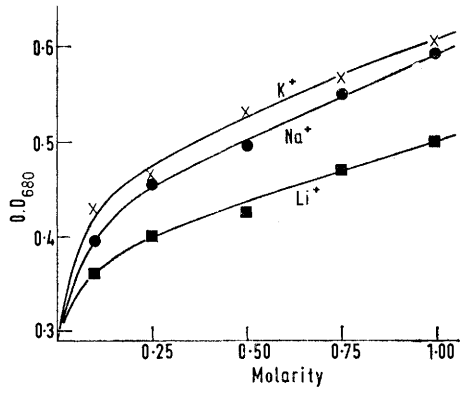

FIGURE 15 Effect of the chlorides of lithium (■), sodium (●), and potassium (×) upon the limiting optical density of carboxymethylamylose at 680 nm and 20°

Zinc sulphate behaved similarly to magnesium sulphate. The action of lithium sulphate is not very different from that of sulphuric acid (Figure 8). The shape of the curve

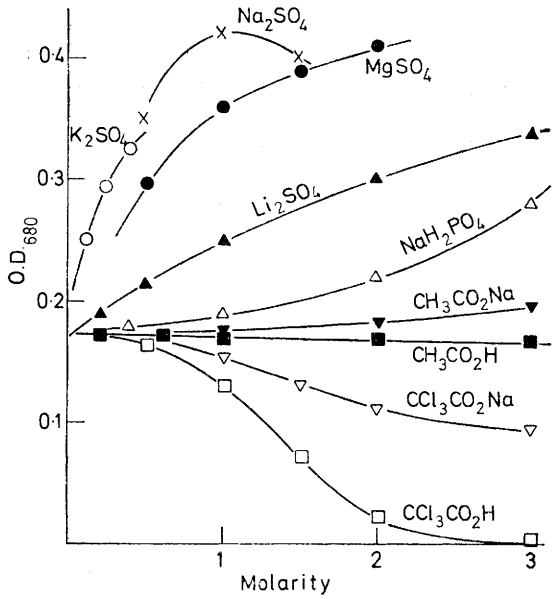

FIGURE 16 Effect of various salts, and of acetic and trichloroacetic acids upon the limiting optical density of starch at 680 nm and 20°

given by sodium dihydrogen phosphate is markedly different from that given by phosphoric acid (Figure 8).

The acetate ion is known [48] to be bound by the Schardinger α-dextrin in the same way that iodine is bound, and is therefore a competitive inhibitor. Its failure to diminish the optical density provided reassurance that an adequate excess of iodine was being used in the experiments.

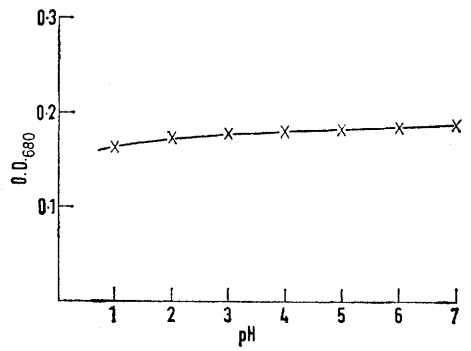

FIGURE 17 Effect of pH upon the limiting optical density of starch at 680 nm and 20°

The trichloracetate ion is much too large to enter the helix, and it is therefore impossible to attribute its action to competitive inhibition. On the other hand, it is reasonable to expect that it would be a weak electrostrictor. Trichloracetic acid was examined because its low pK and powerful action as a protein denaturant suggested that it would form hydrogen bonds with the ring and glycoside oxygen atoms in its undissociated form.

TABLE 2

Comparison of the effect of different cosolutes upon the length (L) of strips of starch gel with their effect upon the iodine-staining power (blue-value, B) of starch in solution at 20°. The salting-out parameter, k_s, refers to benzene in the corresponding electrolyte at 25°, and is taken from the data of McDevit and Long.[45] In the last column, k_s has been multiplied by C_s, the molarity of the electrolyte, to give the logarithm, to the base 10, of the activity coefficient of benzene in the corresponding solution [equation (9)]. ΔL and ΔB refer to increments in L and B, and are expressed as percentages of L and B in water

Cosolute	ΔL (%)	ΔB (%)	k_s	k_sC_s
2M-H_2SO_4	−5.2	+84	+0.26	+0.52
4M-H_2SO_4	−5.2	+97	+0.26	+1.04
5M-H_3PO_4	−9.7	+78		
5M-HCl	+1.4	+9	+0.048	+0.24
5M-HBr	+3.9	−76	+0.007 [b]	+0.035
5M-HI	−17.9	−70 [a]	±0.001 [b]	±0.005
5M-CCl_3CO_2H	−3.0	−99		
5M-CH_3CO_2H	−3.0	−10		
1M-Na_2SO_4	−28.5	+127	+0.548	+0.548
3M-NaH_2PO_4	−32.6	+43		
4M-KF	−9.8	+164	+0.224 [b]	+0.896
4M-KCl	−6.2	+26	+0.166	+0.664
4M-KI	+2.3	−71	+0.065 [b]	+0.260
5M-NaCl	−13.9	+35	+0.195	+0.975
2M-NaBr	−2.0	−64	+0.155	+0.310
4M-LiCl	−5.7	−35	+0.141	+0.564
4M-LiI	+6.3	−100	+0.041 [b]	+0.164
60% w/v sucrose	−13.3	0		

[a] Approximate value; the dark brown colour of HI prevented accurate measurement. [b] Calculated from the data of McDevit and Long [45] on the basis of the principle of additivity.[45]

Control of pH.—In Figure 17, the iodine-binding capacity of starch is shown as a function of pH. Hydrochloric acid

was used to obtain pH 1.0 and 2.0. Sodium acetate–acetic acid buffers (0.2M) were used to obtain pH 3.0, 4.0, and 5.0, because of their negligibly small effect upon the reaction (Figure 16). Sodium phosphate buffers were used to obtain pH 6.0 and 7.0, because of their weak effect upon the reaction at the low concentration (0.1M) needed (Figure 16).

The pH of all salt solutions studied in this work was between 5 and 7, except for the fluorides, which give a slightly alkaline reaction (*ca.* pH 7.5). In these cases, the pH was adjusted to 6 with acetic acid, which was chosen because of its weak effect upon the reaction (Figure 16).

Effect of Salts and Acids upon the Volume of a Gel prepared from Starch with Epichlorohydrin.—The relevant data are collected in Table 2. For comparison, salting-out parameters (k_s) for benzene are included. Benzene, of course, is only a model, and the absolute values of k_s will be different for other non-polar solutes. Analysis showed that the strips of gel were freely permeable to the ions.

DISCUSSION

The interpretation of the present data is essentially a problem in the understanding, at the molecular level, of primary salt effects. For polar non-electrolytes such as starch, this is not a simple undertaking, and there has been no major advance in theory since Long and McDevit [46] published their notable review. In qualitative terms, however, the effect of a salt upon the activity coefficient of a weak organic acid or base can be regarded as the net result of two separate phenomena.

The first of these arises simply out of the fact that the molecules occupy space, and it applies to polar and non-polar molecules alike.[45] When a salt is added to water, the water molecules usually (unless both ions are very large) become not only orientated, but also compressed in the hydration shells of the ions. This usually results in a decrease in volume, and any relatively large molecule is physically 'squeezed out' of solution.[45] The mechanism is not unlike that whereby large pebbles in a box of sand tend to rise to the surface, when it is shaken to facilitate close-packing under gravity.

The second phenomenon occurs with polar molecules only, and is a result of a direct interaction between the polar groups and the water molecules in the hydration shells of the anions and cations. The water-orientation hypothesis that is outlined here, and examined in detail in Part II,[1] is an expanded form of ideas put forward many years ago by Kruyt and Robinson [58] and Meyer and Dunkel,[59] and which were regarded by Long and McDevit [46] as providing the most likely explanation for numerous examples of salting-in, and for reversals in the salting-out order expected from volume changes alone.[46]

For highly polar compounds, there may be a third effect, namely, direct ion–dipole interaction, but this does not appear to be important for most weak organic acids and bases,[46] and reasons have been given [1] for believing that it is also unimportant for carbohydrates in the presence of closed-shell ions.

Ordinarily, a primary salt effect is regarded as a simple effect of salt upon the activity coefficients of the reactants, leading to a change in the rate of a reaction or the position of an equilibrium. It does not normally imply a change in the stoicheiometry of the reaction or the identity of the product. The behaviour of polyvinyl alcohol with iodine (Figure 6) is a typical, primary salt effect. The constancy in the absorption spectrum (Figure 7) indicates that the product is independent of the medium, and the adsorption isotherms give no indication of a change in stoicheiometry (Table 1).

For starch which is not already saturated with I_3^-, it is clear that similar effects must also be operative, but the development of different plateaux or maxima (Figure 3) and the dramatic spectral changes (Figure 4) prove that something else is also happening. If starch were a rigid molecule, with a fixed number of binding-sites, these additional changes could not occur, and one must therefore conclude that the salts are bringing about some kind of conformational change, leading to a change in the number of sites that are able to bind iodine strongly.

The absorption spectra of the chosen sample of starch in 4M-potassium iodide, water, and 4M-potassium fluoride (Figure 4) closely resemble the spectra, in water, of highly branched, moderately branched, and slightly branched fractions of starch,[16] respectively. This strongly indicates that the binding sites that are lost in 4M-potassium iodide, or gained in 4M-potassium fluoride, are simply subtracted from, or added to, existing sequences of binding sites formed from linear segments of the chains. Otherwise expressed, the blocks of I_3^- ions, polymerised in helical segments of the chains, must shrink or grow in length, in response to changes in the medium. On the other hand, they do not shrink or grow when the concentration of I_3^- is changed by less than an order of magnitude (Figure 5).

It is impossible to explain these phenomena as an effect of the medium upon the activity coefficients of I_3^-, the starch molecule as a whole, or the complex as a whole. It is therefore necessary to assume that there is more than one kind of binding site, and that a change in the medium causes their activity coefficients * to change, not only in the absolute sense, but also *relative to one another*.

The idea that this should happen is not more surprising than the well known fact that the salting-out parameters of small molecules depend upon their size, shape, and polarity.[46] The effect is nevertheless remarkably large, and, in order to explain the relative inertness of the system to large changes in the concentration of I_3^-, it would be necessary to assume that the activity coefficients change, relative to one another, by

* It is operationally meaningless to refer to the 'activity coefficient' of a part of a molecule, because it cannot be measured separately, but it is of heuristic value to discuss the behaviour of polymers in terms of the familiar properties of small molecules.

[58] H. R. Kruyt and C. Robinson, *Proc. Acad. Sci. Amsterdam*, 1926, **29**, 1244.

[59] K. H. Meyer and M. Dunkel, *Z. phys. Chem.* (*Frankfurt*), 1931, 553.

at least two orders of magnitude. This is outside the normal range of experience for small molecules,[46] but the idea gains credibility when it is remembered that, in order to create even a single binding site, six contiguous glucose residues have to take up the correct rotameric forms. The concept that, in a co-operative process, the cumulative effect of many small changes in free energy can produce a very large effect overall, has been discussed quantitatively by Flory.[60]

The facts can be explained by assuming that the relative change in activity coefficients occurs either in the free starch molecule, or in the complex, once it is formed, but not in both to the same extent. Of these two possibilities, the former seems much more likely, because the binding sites, once they are occupied, should be structurally identical. The blocks of polymerised I_3^- ions do, of course, differ in length, and therefore also in charge, but the optical density at 680 nm measures only the longest blocks, whose charge densities should all be very similar.

The remainder of the work consisted in an attempt to determine whether the inferred conformational change in the free starch molecule represented an effect of the ions upon the apolar methine and methylene groups, the amphoteric hydroxy-groups, or the basic ring and glycoside oxygen atoms. The results are not more than indicative, but no significant relationship was found between iodine-binding capacity and typical salting-out parameters for non-polar compounds, or the adopted criterion of general solvation (Table 2).

With the notable exception of the results obtained for fluorides, the order of effectiveness of different cations in increasing the iodine-binding capacity (Figures 10—13) is strongly reminiscent of typical salting-out orders for weak organic *bases*.[46] This, together with the direct measurements that have been made on the anomeric effect itself,[1,2] and on the salting-in and salting-out of methyl cellulose,[1] suggests that the iodine-binding capacity is a rather specific function in the state of hydration of the ring and glycoside oxygen atoms alone.

It is therefore suggested that the main driving force behind the conformational change is the *exo*-anomeric effect, and that the explanation for the present results must be sought mainly in the idea that the glucose residues in the starch and derivatised amylose differ widely in the extent to which the A_1 rotameric form is sterically preferred.

In the longer chains, short-range interactions of the kind considered in the theoretical section probably favour the A_1 form sufficiently strongly that most glucose residues are already in this form. There would then be little difference in polarity between the free and complexed binding sites, so that any effect of salts upon the position of the equilibrium would be minimal.*

In the vicinity of branching-points or substituents, longer range interactions probably intervene to such an extent that the preference for the A_1 form is diminished to 1 kcal mol^{-1} or less, giving rise to a significant proportion of A_2 forms at equilibrium. The mean difference in polarity between the free and occupied binding sites would then be greater, so that changes in the medium would have a greater effect upon the activity coefficient of the free binding-sites compared to those that are occupied.

The direction of the proposed changes can be predicted from the water-orientation hypothesis. For example, with any given anion (except F^-), a change in the cation from Na^+ to Cs^+ would increase the activity coefficient of the more polar, A_2 form compared to the less polar, A_1 form, simply by dehydrating O(1) and O(5); this would then displace the equilibrium towards the A_1 form, favouring complex formation. On the other hand, a change from Na^+ to Li^+ would hydrate O(1) and O(5), and decrease the activity coefficient of the A_2 form relatively to that of the A_1 form, disfavouring complex formation.

The interesting question as to whether a monodisperse fraction of pure amylose would, or should, behave in the same way as the materials investigated here has been raised by the referees of this paper, who emphasised the difference between binding sites near the ends of chains, and those at the centre, and pointed out that even an infinitely long chain would still contain different binding sites, if the system were not in rapid thermodynamic equilibrium. It would be very difficult to obtain a definitive experimental answer to these questions, because of the difficulty of preparing suitable fractions that would also give stable solutions. A useful observation can, however, be mentioned: in selecting suitable materials for the present work, it became reasonably clear that the *sensitivity* of the limiting iodine-binding capacity to changes in the medium increased with increasing degree of branching or substitution. We therefore consider that it is correct to attribute the present findings largely to these sources of heterogeneity.

The behaviour of the fluorides remains obscure. One possibility is that, when the A_1 form is already strongly stabilised, other salt effects become important in influencing the completion of helix formation, by rotation about the O(4)–C(4) bonds. However, it must be remembered that fluorides are not considered to be strong electrolytes, and are thought to exist partly as ion-pairs in aqueous solution;[61] the consequences of this are hard to predict in the present context.

In a sense, the experiments carried out on the ionic derivatives of amylose (Figures 9, 14, and 15) raise more questions than they answer, because the possibility of specific, ion–ion interactions must also be considered. It is, however, interesting that the presence of ionic

* It is not suggested that free amylose is already helical in solution under the conditions of these experiments. The question of rotation about O(4)–C(4) is not being considered here.

[60] P. J. Flory, 'Statistical Mechanics of Chain Molecules,' Wiley–Interscience, New York, London, Sydney, and Toronto, 1969, p. 286.

[61] H. S. Harned and B. B. Owen, 'The Physical Chemistry of Electrolytic Solutions,' Reinhold, New York, 1950, 2nd edn., pp. 383—385.

groups in amylose, apart from bringing about a possible increase in the sensitivity to counterions, introduces no fundamentally new feature that is not already fairly demonstrable in the neutral starch molecule. This result should be useful in connection with any discussion of the conformation of anionic polysaccharides in aqueous solution.

Recent developments in other laboratories are consistent with the present conclusions. Banks *et al.*[62] have studied the effect of inorganic salts and alkalis upon the viscosity of amylose in aqueous solution, and their results are complementary with those reported here. From a theoretical analysis of the optical rotations of α-linked disaccharides, Rees and Scott [63] concluded that the *exo*-anomeric effect is able to override steric preferences in these compounds. Most recently, Lemieux and Koto [64] have adduced new evidence, from ^{13}C n.m.r. spectroscopy, which strongly suggests that steric preferences are not normally too large for the *exo*-anomeric effect to have an important influence upon conformation. If this is generally true, it is evident that the *exo*-anomeric effect must be a phenomenon of very considerable, if not profound, biological significance.

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[62] W. Banks, C. T. Greenwood, D. J. Hourston, and A. R. Procter, *Polymer*, 1971, **12**, 452.

[63] D. A. Rees and W. E. Scott, *J. Chem. Soc.* (*B*), 1971, 469.

[64] R. U. Lemieux and S. Koto, *Tetrahedron*, 1974, **30**, 1933.