625. *The Action of Ionizing Radiations and of Radiomimetic Substances on Deoxyribonucleic Acid. Part VI.* Physicochemical Measurements of the Action of Bischloroethylmethylamine.*

By J. A. V. Butler, L. Gilbert, and D. W. F. James.

The mobilities and sedimentation and diffusion constants of deoxyribonucleic acid, which has been treated with bischloroethylmethylamine, have been determined after various periods of reaction. The molecular size decreases for a considerable time after the initial reaction. The initial change of viscosity appears to be independent of this, at least at pH 7, and is ascribed to changes of interaction and configuration consequent on the breakage of hydrogen bonds owing to the alkylation of the primary amino-groups of the bases.

It has been shown previously (Butler and Smith, *J.*, 1950, 3411) that bischloroethylmethylamine and similar substances destroy the characteristic viscosity of deoxyribonucleic acid solutions and the change has been shown (Conway, Gilbert, and Butler, *J.*, 1950, 3421) to be accompanied by a large decrease in the apparent molecular weight deduced from sedimentation and diffusion measurements. The interpretation of these observations has been difficult because of lack of knowledge of the molecular state of the nucleic acid in these solutions, which are anomalous in many respects, in consequence of which it has not been known if the sedimenting and diffusing unit is a single molecule or an association of several and the size and shape have been in doubt. There is undoubtedly marked molecular interaction at the concentrations (*ca.* 0·05%) at which investigations of this kind are possible and it is difficult to distinguish if the effects are due to (1) a decrease of interaction between the elementary particles, (2) changes in the shape of the particles, similar to those which have been shown to occur with flexible polyelectrolytes on neutralization and addition of salts (*e.g.*, by Fuoss and Maclay, *J. Polymer Sci.*, 1951, **6**, 305; Jordan, *Trans. Faraday Soc.*, 1950, **46**, 792), or (3) a real decrease in particle size.

When the nucleic acid is treated with the amine some reaction with the phosphate "backbone" probably occurs, with the consequent partial neutralization of the nucleic acid anion, which might produce effects on the viscosity similar to those found when the ionized groups of flexible polyelectrolyte chains are neutralized. In order to find if such effects are adequate to explain the loss of viscosity of nucleic acid we determined its mobilities and sedimentation constants in the original state and after treatment with the amine, and we have also observed the change of these properties during the reaction. Although the absolute determination of charge is difficult, it was expected that it would be possible to infer from the mobilities whether there was a diminution of charge sufficient to produce the observed decrease of viscosity.

* Part V, *J.*, 1952, 834. This paper, and Part IV (*J.*, 1952, 626), were read in abstract at the XIIth International Congress of Pure and Applied Chemistry, New York, Sept., 1951.

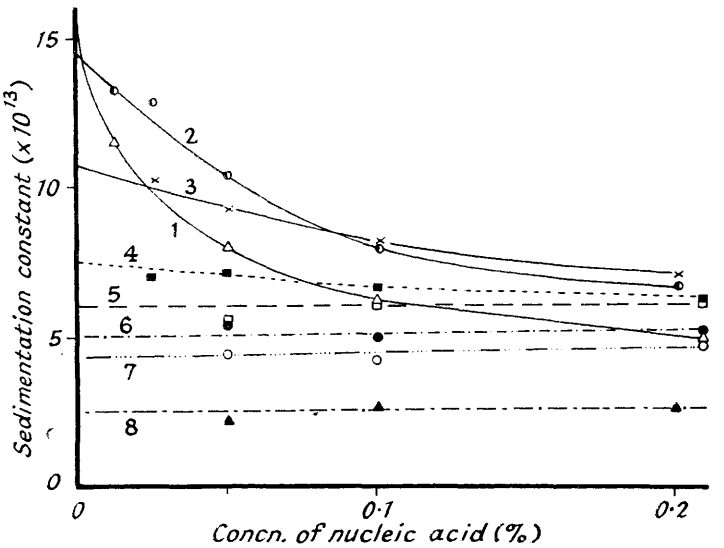
The mobilities observed with nucleic acid (G 2) and the same material treated with the amine (G 2/1) in a variety of buffer solutions are shown in Table 1.

TABLE 1. *Mobilities of nucleic acid and nucleic acid after treatment with the amine (ascending boundaries)* (20°) ($\times 10^5$ *cm.*^{2}*/sec.* v).

pH	4·5	5	6	7	8	8·5
Nucleic acid (G 2)	16·2	16·2	—	16·1	16·8	20·4
Nucleic acid (G 2) treated with amine	10·6	11·45	12·0	12·3	16·0	—

The proportion of the phosphate groups of the nucleic acid which have reacted with the amine and therefore become incapable of being ionized is unknown. The total amount of the amine which has reacted in our experiments was, however, known to be about 1 residue per 4 atoms of phosphorus, and since it has been shown that a part of this is combined with the bases of the nucleic acid (Press and Butler, *J.*, 1952, 626) the amount combined with the

FIG. 1. *Sedimentation constants as function of nucleic acid concentration after treatment with the amine for various times in sodium hydrogen carbonate. Reaction carried out with nucleate concentration* 0·2% *and amine concentration* 0·2%.



1, *Original nucleic acid.* 2, *After* 1 *day.* 3, *After* 3 *days.* 4, *After* 5 *days.* 5, *After* 7 *days.* 6, *After* 7 *days* + 7 *days' dialysis at* 0°. 7, *After* 18 *days, no dialysis.* 8, *After* 7 *days* + 7 *days' dialysis and freeze-drying.*

phosphate groups is unlikely to exceed half a residue of amine per tetranucleotide. The maximum possible degree of neutralization of the charged phosphate groups is thus not likely to exceed 25%.

It can be seen that the mobility of the product (G 2/1) is somewhat less than that of the parent nucleic acid at all pH's below 8.

Quantitative interpretation of these results is complicated by the possible variation of the frictional resistance to motion of the particles in the two cases. This can be eliminated by introducing the sedimentation constants in the two solutions, which have been measured in identical solutions at pH 7 only. Combining the equations $M(1 - V\rho) = fs$, and $\mu_e = ne/f$, where M is the mass of the sedimenting particle, V its specific volume, ρ the density of the solvent, f the frictional resistance, s the sedimentation constant, μ_e the electrophoretic mobility, and n the effective number of unit charges e on the particle, we obtain

$$ne/M = (1 - V\rho)\mu_e/s$$

from which the ratio of the *effective* charge to mass (ne/M) of the particle can be determined.

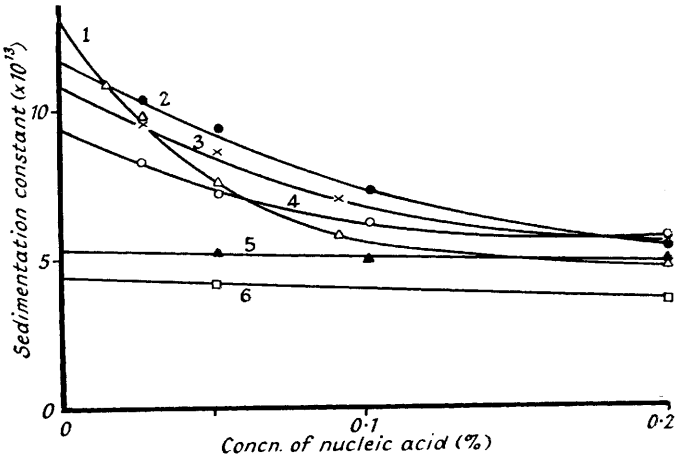
This calculation for the two products at 0·2% in 0·1M-phosphate buffer (pH 7) is given below:

	$10^{13}s$	$10^{5}\mu_e$	$10^{10}ne/M$
Nucleic acid (G 2)	5·88	16·2	3·72
Nucleic acid (treated with amine and freeze-dried)	2·31	12·4	7·25

It is evident that the "effective charge" on unit mass of the material, at this pH, so far from being diminished by the reaction, is increased. The "effective charge," however, includes the charges of those counter ions which are carried along by the particles in their electrophoretic migration. The relation between the effective charge and the actual or total possible charge of particles is, in general, not known. It can only be inferred that the existence of a net effective charge implies the presence in the molecules, unless specific adsorption of ions of opposite sign is present, of more ionizable groups of the same sign.

In order to find to what extent the loss of viscosity is due to the reduction of molecular

FIG. 2. *Sedimentation constants as function of nucleic acid concentration after treatment with amine for various periods in phosphate buffer* (M/10) *at* pH 7. *Reaction carried out with nucleate concentration* 0·2% *and amine concentration* 0·2%.



1, *Control nucleic acid.* 2, *After* 1 *day.* 3, *After* 4 *days.* 4, *After* 7 *days.* 5, *After* 7 *days* + 7 *days' dialysis at* 0°. 6, *After* 14 *days.*

weight of the nucleic acid in the reaction, we determined the sedimentation constants at different times after the addition of the amine to nucleic acid in buffer solutions.

The results of these determinations, made in each case at three concentrations of nucleic acid, for the reaction in 0·1N-sodium hydrogen carbonate, are shown in Fig. 1. It can be seen that the initial effect is the disappearance of the marked concentration dependence of the sedimentation constant, combined with a decrease in the limiting value at zero concentration. This is followed by a slow downward drift of the sedimentation constants which continues for a considerable time. Since it was thought that this slow change might be due to a slow hydrolysis of the phosphate–sugar links by sodium hydrogen carbonate (pH *ca.* 8·8), similar observations were made in a phosphate buffer of pH 7 (Fig. 2). A similar sequence of events occurs, but rather more slowly, and the final sedimentation constants observed are appreciably higher than those reached in sodium hydrogen carbonate in the same time.

These decreases of sedimentation constant could be due either to decreases in molecular size or to changes of shape resulting in an *increased* resistance to motion. The fact that final molecular weights observed (from sedimentation and diffusion; *J.*, 1950, 3421) in preparations which had remained in solution for 2 weeks after the addition of amine are much lower than those of the original nucleic acid suggests that a real decrease of molecular

TABLE 2. *Diffusion and sedimentation constants of deoxyribonucleic acid during reaction with the amine.*

Treatment	Concn. (%)	Diffusion const. ($\times 10^7$) D_B	D_M	Sedimentation const. ($\times 10^{13}$)	Mol. wt.
G_3 treated with amine (0·4%) in 0·1N-sodium hydrogen carbonate (2 days) ...	0·2	1·55	1·60	7·1	
	0·1	1·74	1·41	8·2	
	0·05	1·34	1·50	9·3	
	0·0	1·54 *	1·50 *	10·8	4×10^5
G_3 treated with amine (0·4%) in 0·1M-phosphate buffer at pH (2 days)	0·2	1·4	1·4	5·4	
	0·1	0·91	1·0	7·1	
	0·05	0·73	0·77	9·0	
	0·0	0·55 †	0·66 †	11·5	1×10^6
G_1 treated with amine in sodium hydrogen carbonate for 14 days and freeze-dried (G 2/1)	0·2	4·5	—	2·5	
	0·1	6·4	—	2·5	
	0·05	7·0	—	2·5	
	0·0	8·0	—	2·5	$1·7 \times 10^4$
S similarly treated (S 1/1)	0·2	4·6	—	2·0	
	0·1	5·2	—	2·4	
	0·05	5·9	—	—	
	0·0	7·5	—	3·0	$2·2 \times 10^4$

* Mean of figures at the different concns. † Extrapolated to zero concn.

weight occurs. This has been confirmed by some new observations given in Table 2. Measurement of the diffusion coefficients at an early stage of the reaction is difficult since appreciable changes may occur during the experiment. An attempt to determine the diffusion coefficient after 2 days' reaction, followed by equilibration with the buffer by

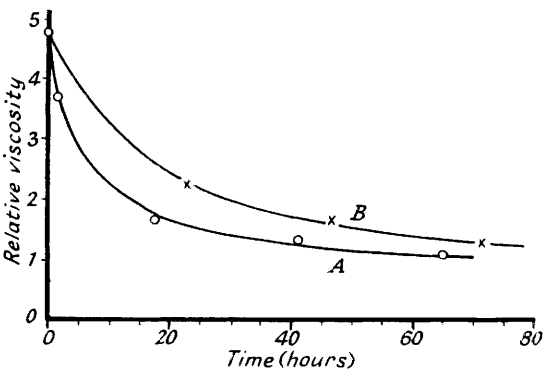


FIG. 3. *Change of viscosity with time of* 0·4% *thymonucleic acid with* 0·4% *of the amine in* (*A*) 0·1N-*sodium hydrogen carbonate and* (*B*) 0·1M-*phosphate buffer* (pH 7). *Viscosity determined after dilution to* 0·1%.

dialysis for 1 day, has given the results shown in Table 2. In view of the time taken by the diffusion experiments it has seemed desirable to use the sedimentation constant after 3 days with the diffusion constant so determined. The difference between the sedimentation constants after 2 and 3 days is, however, not large. No great reliance can be placed on the numerical values owing to the difficulties involved but they clearly indicate that while the diffusion constants are somewhat increased in the earlier stages of the reaction in the bicarbonate solution, in 2 days the sedimentation and diffusion constants in phosphate are little changed, although the viscosity has diminished considerably. Fig. 3 shows the changes of viscosity in the two cases. It follows from this that very little change of molecular weight occurs during the initial decrease of viscosity in the phosphate buffer, but that a significant decrease of molecular weight occurs in the same time in the bicarbonate buffer. The rate of change of viscosity is, however, greater in the sodium hydrogen carbonate solution and it is possible that the difference between the two cases would not be very great if the determinations were made at the same viscosity rather than at equal times.

Experimental

Sedimentation and Diffusion Measurements.—The sedimentation rates were determined in the Spinco ultra-centrifuge at *ca.* 60,000 r.p.m. They are corrected to 20°. The diffusion measurements were made by examining the Schlieren diagram of the boundary between the solution and solvent in the Perkin–Elmer diffusion apparatus at various periods after the making of the boundary. The boundary was initially made by sliding the upper part of the electrophoresis cell into position and then removing the boundary into an observable position by slow displacement. Sharp initial boundaries were obtained in this way. Calculations of D from the height (D_H) and from the breadth (D_M) of the Schlieren diagram at its inflection point were in good agreement (for details, see Alexander and Johnson, "Colloid Science," Oxford Univ. Press, 1949, Chap. X). The viscosity measurements were as described by Butler and Smith (*loc. cit.*).

Mobilities.—The mobilities were determined in the electrophoresis apparatus after careful equilibration of the solutions by dialysis with the buffers in which they were to be measured. The conductivites and pH's of the dialysed solutions and the buffers were determined to ascertain if complete equilibration had been achieved. The buffer solutions were made up to 0·1 ionic strength, as given by Miller and Golder (*Arch. Biochem.*, 1950, **29**, 420). Since the mobility might diminish in the early stages of the reaction and increase to about its initial value in the subsequent stages, an attempt was made to determine the mobility at intervals during the reaction at pH 7·4, where the final mobility is nearly the same as the initial. In order to do this, samples of the reaction mixture, taken at intervals, were dialysed against the buffer used (0·0625M-disodium hydrogen phosphate, 0·02M-sodium dihydrogen phosphate) for 3 hours only with constant agitation. The results given below (0·2% of the nucleic acid S/1) showed no significant change during the reaction :

Time from start of reaction (hr.)	0·0	9·5	29	96
Mobility ($\times 10^5$)	14·4	14·3	14·4	14·8

The nucleic acid (S) used here has a rather smaller mobility at the given pH than that referred to in Table 1.

"Amine-treated" Nucleic Acid.—The preparation of the samples G 2/1 and S 1/1 is described by Press and Butler (*loc. cit.*).

Discussion

It was expected that the effective (negative) charge of the nucleate ion would be diminished by the reaction with the "nitrogen mustard," owing to the partial esterification of the phosphate groups. The effective charge was found to be increased, so that it must be concluded that the numbers of counter ions carried by the particles is diminished to a greater extent than the charge on the nucleate ions themselves. This may well happen if considerable changes of molecular size or configuration occur.

It is quite clear that there is no neutralization of the charge of the nucleate ions which would be sufficient by itself to account for the loss of viscosity, such as occurs when flexible polyelectrolytes are neutralized. However, it has been shown that 25% neutralization of the latter causes very little change of viscosity (Arnold and Overbeck, *Rec. Trav. chim.*, 1950, **69**, 192; Oth and Doty, *J. Phys. Chem.*, 1952, **56**, 43). Thus the loss of viscosity cannot be a simple consequence of the partial neutralization of the nucleate ions.

We must enquire how far it is due to the observed decreases of molecular weight. In sodium hydrogen carbonate solutions, some decrease of molecular weight occurs while the viscosity is decreasing, but in the phosphate buffer it appears that molecular weight changes very little during this period. It therefore does not seem possible to account for the loss of viscosity, at least in the phosphate buffer, as due to the breakage of the elongated molecules of nucleic acid.

The characteristic high viscosity of nucleic acid solutions is due mainly to the interaction of the nucleic acid particles with each other, giving rise even at small concentrations (>0·01%) to something resembling a gel-like net-work (Pouyet, *J. Chim. phys.*, 1951, **48**, 616; Benoit, *ibid.*, p. 612). The decrease of viscosity brought about by salts at small concentrations can be attributed mainly to the decrease of such interactions, as is shown by the change in the nature of the diffusion patterns brought about by salts (Butler and James, *Nature*, 1951, **167**, 844). It is not known with certainty how the interaction

between the particles occurs, but the fact that it is greatly reduced by even small salt concentrations would indicate that it is mainly electrostatic in character. Since reaction with the amine does not modify the electrical character of the particles very much, it is difficult to account for a marked change of viscosity in this way, although such effects may contribute.

However, the interaction of the particles may be modified as the result of the reaction for another reason. It is well known that the viscosity of nucleic acid solutions is diminished, to some extent irreversibly, by agents such as heat, acids, and alkalis (Creeth, Gulland, and Jordan, *J.*, 1947, 1141; Zamenhof and Chargaff, *J. Biol. Chem.*, 1950, **186**, 207; Chargaff, *J. Cell. Comp. Physiol.*, 1951, **38**, 41); and simple denaturing agents, such as urea and phenol, also cause irreversible changes in the nucleic acid (Conway and Butler, *J.*, 1952, 3075). Such irreversible changes have been ascribed to the breakage of intramolecular hydrogen bonds between hydroxyl and primary amino-groups of the bases in the nucleic acid particle, and it has been suggested that nucleic acid is a highly organised structure maintained by such bonds. At least, treatment with these substances gives rise to material with lower viscosities and a more compact configuration.

Since it has been shown (Press and Butler, *loc. cit.*) that the amine reacts with the amino-groups of the nucleic acid, it will necessarily cause a breakage of the hydrogen bonds involving the amino-groups which are alkylated. This is sufficient to account for a loss of viscosity similar in magnitude to that produced by phenol or urea at much higher concentrations. A small number of such acts would be sufficient to reduce considerably the length of the nucleic acid particle. It is possible to understand on this basis the great effect of comparatively small concentrations of the reagent on the physical properties of the nucleic acid. Such a change of the nucleic acid to a more compact configuration would naturally decrease the possibilities of interaction between the nucleic acid particles.

It is evident that even in the phosphate solutions the initial action of the reagent is followed by a continued drift of the sedimentation constants to lower values, which, since it results in lower molecular weights, must be attibuted to a real breakdown of the nucleic acid particles into smaller units. This occurs more quickly and also more extensively in the bicarbonate than in the phosphate solutions and is therefore probably due to a hydrolytic fission of the molecule. The question then arises why nucleic acid which has reacted with the amine is more easily broken down than the original nucleic acid. There are two possible explanations of this behaviour: (1) As suggested by Butler, Gilbert, James, and Ross (*Nature*, 1951, **168**, 985) the tri-esterified phosphate groups, formed by the action of the reagent, are relatively unstable and break down preferentially at the sugar–phosphate bond. The possibility is being investigated further by Dr. W. J. C. Ross. (2) The breakage of the hydrogen bonds between the bases may result in the phosphate–sugar links' being more accessible to attack by hydrolytic reagents.

It will be evident that no simple explanation can be given of the loss of viscosity of nucleic acid solutions when treated with the amine, but that several effects occur each of which is capable of contributing to it. It has been shown that in particular the initial reaction of the reagent brings about (*a*) changes of configuration and (*b*) consequent changes of interaction of the particles with each other, but that at the same time a slow decrease of molecular size of the particles occurs which continues for a considerable time after the initial reaction is completed.

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