590. *The Action of Denaturing Agents on Deoxyribonucleic Acid.*

By B. E. Conway and J. A. V. Butler.

Phenol or urea causes a considerable decrease in the viscosity of solutions of deoxyribonucleic acid, and the effect is irreversible in that the original viscosity is not recovered when the agent is removed by dialysis. Measurements of viscosity, diffusion, and sedimentation indicate that the interaction between the particles is diminished by the presence of the agent, and that when the agent is removed the particles assume a more compact form. It is suggested that this is due to the breakage of the hydrogen bonds to which the configuration of the nucleic acid particles is due.

That urea and other effective protein denaturants reduce the viscosity of solutions of deoxyribonucleic acid was noted by Jenrette and Greenstein (*Cold Spring Harbor Symp.*, 1941, **9**, 236). We have re-examined this phenomenon since it was hoped that it would elucidate the loss of viscosity produced by substances of the "mustard" class at much smaller concentrations (Butler and Smith, *J.*, 1950, 3411), and also provide information on the properties of the nucleic acid. Fig. 1 shows effects of applied pressure on the viscosity of nucleic acid solutions containing urea, as determined in a capillary viscometer. Similar effects are produced by phenol (Fig. 2) which is also known to act as a protein denaturant (Alexander, *Ann. New York Acad. Sci.*, 1951, **53**, 24). The effect of phenol is mainly on the low-pressure viscosity, *i.e.*, the increased viscosity which is observed at low rates of flow is greatly diminished, but the viscosity at higher pressures is not greatly affected. The effect of concentration of the added substance at a constant pressure is shown in Fig. 3. It can be seen that phenol is more effective than urea for a given molar concentration, but the phenomena observed are of course limited by the solubility. Ethanol also reduced the viscosity somewhat, but its main effect is at small concentrations ($<0{\cdot}2$M)—increasing the concentration from 0·2 to 2M has very little further effect.

It has been found that the effects of both urea and phenol on the nucleic acid are irreversible, *i.e.*, the viscosity is not restored by removing the solute by dialysis. In fact the following table shows that the viscosity of the solutions is decreased further when the agent is removed. Only small changes are brought about by dialysis and freeze-drying of nucleic acid solutions in neutral water.

	$\eta_{rel.}$ at 60 mm.
(1) 0·1% Solution of nucleic acid I	14·0
(2) ,, +7% of phenol	10·3
(3) As (2) but after dialysis and freeze-drying	4·9
(4) 0·1% Solution of nucleic acid II	10·5
(5) ,, after dialysis	10·0
(6) ,, after dialysis and freeze-drying	9·2

As is well known, the viscosity of nucleic acid solutions is decreased by small concentrations of salt. This has been stated to be reversible (Greenstein and Jenrette, *J. Nat. Cancer Inst.*, 1940, **1**, 77). It has been found that, in the presence of urea and phenol, the addition of an electrolyte has very little effect on the viscosity (Fig. 4), *i.e.*, urea or phenol produces an effect similar to that of a small concentration of electrolyte. When the phenol or urea is removed by dialysis, the viscosity is again reduced by the addition of salts.

The fact that phenol and urea produce permanent effects on the nucleic acid suggests that their action is similar to the denaturation of proteins. It has been found (cf. Neurath and Saum, *J. Biol. Chem.*, 1939, **128**, 347) that denaturing agents disorganise the configuration of the native protein molecule, probably by the breakage of the hydrogen bonds

(Mirsky and Pauling, *Proc. Nat. Acad. Sci.*, 1936, **22**, 439; Cannan, Kilpatrick, and Palmer, *Ann. New York Acad. Sci.*, 1941, **41**, 2437) which maintain the protein in its original configuration. This is supported, for example, by infra-red absorption spectra (Buswell, Krebs, and Rodebush, *J. Phys. Chem.*, 1940, **44**, 1126; Richards and Thompson, *J.*, 1947, 1248). Denaturing agents in general appear to be substances which are capable of breaking such bonds. This view is supported by previous observations of the action of acids and alkalis on deoxyribonucleic acid (Creeth, Gulland, and Jordan, *J.*, 1947, 1141; Zamenhof and Chargaff, *J. Biol. Chem.*, 1950, **186**, 207) which has been described as denaturation. It was also shown by Gulland, Jordan, and Taylor (*J.*, 1947, 1131) that, when the nucleic acid is titrated with acid or alkali, hysteresis is observed—the titration curves starting from the neutral region differ from those obtained on back-titration from acid or alkaline pH—which was attributed to the titratable amino- and hydroxyl groups' being united in hydrogen bonds and inaccessible to titration until fairly acid or alkaline pH is reached. Chargaff (*J. Cell. Comp. Physiol.*, 1951, **38**, Suppl. 1, 41; *Fed. Proc.*, 1951, **10**, 654)

FIG. 1. FIG. 2.

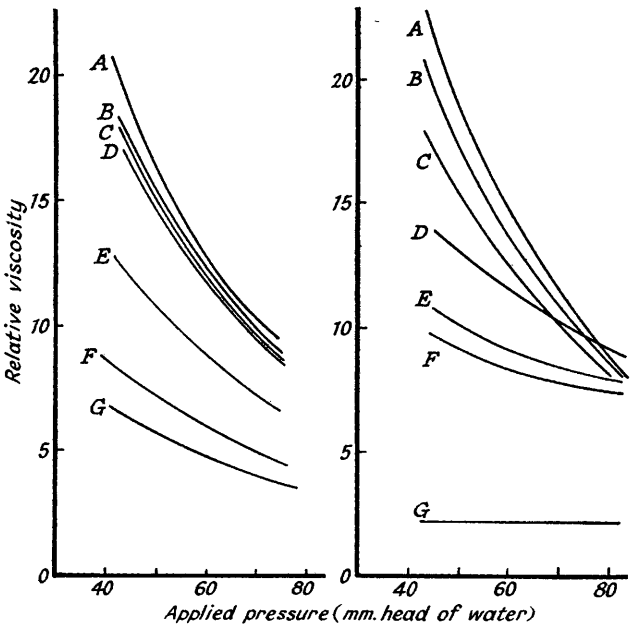

FIG. 1. *Effect of urea on the viscosities of 0·1% deoxyribonucleic acid.*

A, Nucleic acid in water; B—G, nucleic acid in urea solutions, viz.: B, 10^{-3}M; *C,* 10^{-2}M; *D,* 0·1M; *E,* M; *F,* 4·0M; *G,* 7·0M.

FIG. 2. *Effect of phenol on the viscosities of 0·1% deoxyribonucleic acid solutions.*

A, Nucleic acid in water; B—F, nucleic acid in phenol solutions, viz.: B, 0·1%; *C,* 0·5%; *D,* 2·0%; *E,* 5·0%; *F,* 7·0%; *G, nucleic acid in* 7·0% *phenol and* 0·1N-*sodium chloride.*

have shown that in all the deoxyribonucleic acids investigated by them there is a fairly constant balance between primary amino-groups and hydroxyl groups, so that the nucleic acid may be regarded as an organised structure maintained by intra-molecular hydrogen bonds. The effects of reagents such as phenol and urea which break hydrogen bonds may thus be akin to denaturation processes, such as occur with native proteins.

We have endeavoured to characterize the changes brought about by these agents by a study of the viscosity and of other properties in solution.

Viscosities of Nucleic Acid Solutions.—Fig. 5 shows the variation of the reduced viscosity $(\eta-\eta_0)/c\eta_0$ with the concentration of the nucleic acid at a constant shear rate (250 sec.$^{-1}$) under the conditions stated in the legend. For aqueous solutions of untreated nucleic acid over the concentration range studied, the reduced viscosity decreases with increasing concentration, except in 0·1N-sodium chloride where there is a slight trend in the opposite direction. The diminution is much less than that observed with certain flexible polyelectrolytes (Fuoss and Strauss, *Ann. New York Acad. Sci.*, 1949, **41**, 836) and is possibly due to the tendency of elongated molecules to arrange themselves with their long axes in the same direction and, to some extent, to the hydrodynamic conditions of flow of viscous fluids in a capillary tube.

The viscosities at the concentrations in question are largely determined, in the absence of salt, by intermolecular interactions (cf. Butler and James, *Nature*, 1951, **167**, 844). It has been shown by Pouyet (*J. Chim. phys.*, 1951, **48**, 90) and Benoit (*ibid.*, p. 86) that these interactions persist in nucleic acid solutions to concentrations of the order of 0·001%; and, although they are diminished by salts, they probably still occur to some extent in the

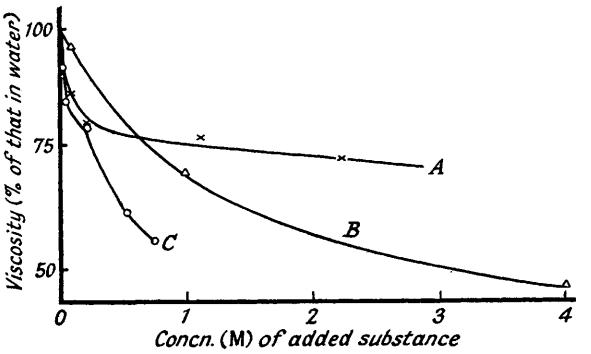

FIG. 3. *Effect of concentration of the added substances on the viscosity of nucleic acid.*

A, Ethanol.
B, Urea.
C, Phenol.

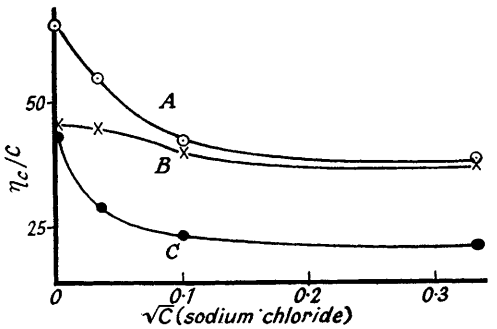

FIG. 4. *Effect of sodium chloride concentration on reduced viscosity of deoxyribonucleic acid* (0·05%) *at constant shear rate* (250 $sec.^{-1}$).

A, Nucleic acid alone.
B, Nucleic acid in 6M-*urea.*
C, Nucleic acid after treatment wth 6M-*urea, followed by dialysis.*

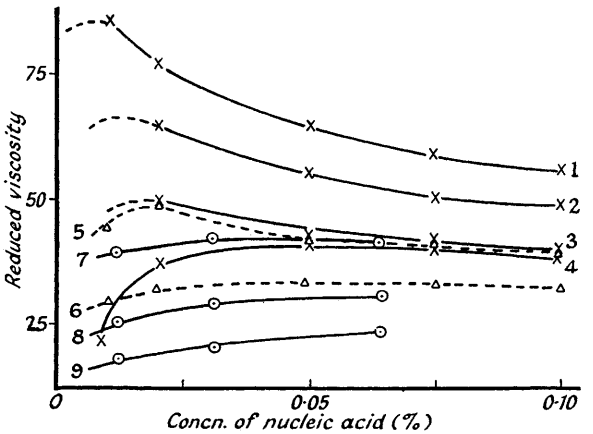

FIG. 5. *Specific viscosities of deoxyribonucleic acid solutions at a constant rate of flow.*

In water : 1, *water only ;* 2, 0·001N-NaCl; 3, 0·01N-NaCl; 4, 0·1N-NaCl.

In 6M-*urea :* 5, 6M-*urea only ;* 6, 6M-*urea* + 0·1N-NaCl.

In water after treatment with 6M-*urea and dialysis :* 7, *water only ;* 8, 0·001N-NaCl; 9, 0·1N-NaCl.

salt solutions used by us. The reduced viscosities found are therefore considerably greater than those at zero concentration. It is impossible in these circumstances to measure, with a capillary viscometer, solutions which are sufficiently dilute to have intrinsic viscosities approaching those at zero concentration; the information about molecular shape and dimensions which can be obtained from our measurements is thus limited. It can be seen, however (Fig. 5), that the effect of urea on the viscosity–concentration curve at constant rate of flow is very similar to that of sodium chloride and we may conclude that its presence mainly influences the interaction of the particles. After treatment with urea followed by

dialysis, the sensitivity of the material to salt concentration is similar to that of the untreated nucleic acid; by a comparison with the original nucleic acid, this must be ascribed to a diminution of interactions between the particles, produced by the salt, but it is evident that the particles have also been permanently modified in some way during the removal of the urea. Replacement of the urea after its removal does not restore the viscosity.

The character of the changes brought about by urea is shown by the effect of rate of flow on the viscosity. These are plotted in Fig. 6 for a constant nucleic acid concentration. It can be seen that, while the viscosities measured are a function of the rate of flow both in aqueous solution and in the presence of urea, the dependence on flow rate is largely lost when the urea is removed. The simplest explanation is that the particle becomes more symmetrical when the urea is removed, and this is supported by diffusion and sedimentation observations which are given below.

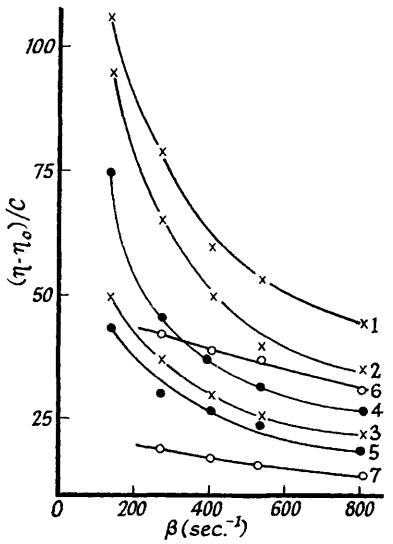

FIG. 6. *Reduced viscosities of nucleic acids* (0·02%) *as a function of the rate of flow.*

Nucleic acid in water: 1, *alone;* 2, 0·01N-NaCl; 3, 0·1N-NaCl.

Nucleic acid in 6M-*urea:* 4, *alone;* 5, 0·1N-NaCl.

Nucleic acid after treatment with 6M-*urea, followed by dialysis:* 6, *water;* 7, 0·1N-NaCl.

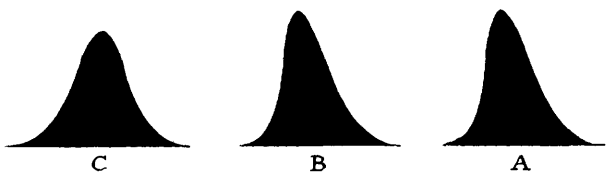

FIG. 7. *Schlieren diagrams of boundary in diffusion of nucleic acid* (0·2%). *Diffusion is occurring from right to left.*

A, In 0·1N-NaCl.
B, In 6M-*urea* + 0·1N-NaCl.
C, In 0·1N-NaCl, *after treatment with* 6M-*urea, followed by dialysis.*

Sedimentation and Diffusion.—Measurements of sedimentation and diffusion constants have been made of nucleic acid in water, 6M-urea, and 7% phenol solution, and of nucleic acid which has been treated with urea and recovered by dialysis in water, in all cases in the presence of 0·1N-sodium chloride. The results are shown in the annexed Table. The significance of the diffusion values is subject to considerable uncertainty owing to the asymmetric nature of the boundary curves (cf. Butler and James, *Nature*, 1951, **167**, 844). The asymmetry remains in the presence of phenol and of urea (Fig. 7); but the boundaries obtained with the substance which has been treated with urea and dialysed are quite symmetrical. In the sedimentation, the sharp boundary characteristic of nucleic acid is maintained in the presence of urea and phenol, but in the dialysed material a broader band is observed which spreads more rapidly than is to be expected from the diffusion constant. This is evidence of the heterogeneity of this material with respect to sedimentation constant. From the figures (Table) it is seen that on the whole the values obtained in the presence of phenol and urea are not greatly different from those in water. On the other hand the

Sedimentation (S) and diffusion (D) constants of nucleic acid, in the presence of 0·1N-*sodium chloride.*

Solution		Concn. of nucleic acid, % 0·05	0·1	0·2	M	f/f_0	
Water	S	9·9	7·4	5·8	—	—	—
	D	4·65	4·00	4·05	$1·5 \times 10^6$	6·9	316
Aq. phenol (7%)	S	11·1	8·75	5·4	—	—	—
	D	—	5·1	3·7	—	—	—
6M-Urea	S	8·5	6·5	6·2	—	—	—
	D	—	—	—	—	—	—
,, after dialysis	S	14·7	11·6	—	—	—	—
	D	9·1	6·3	—	$7·5 \times 10^5$	3·35	50

nucleic acid which has been treated with 6M-urea and dialysed has values of both S and D greater than those of the nucleic acid itself. This means that the molecular weight is of the same order in the two cases, but that the frictional resistance to sedimentation or diffusion, which will occur if the molecule is more compact, is less in the latter. The Table gives the

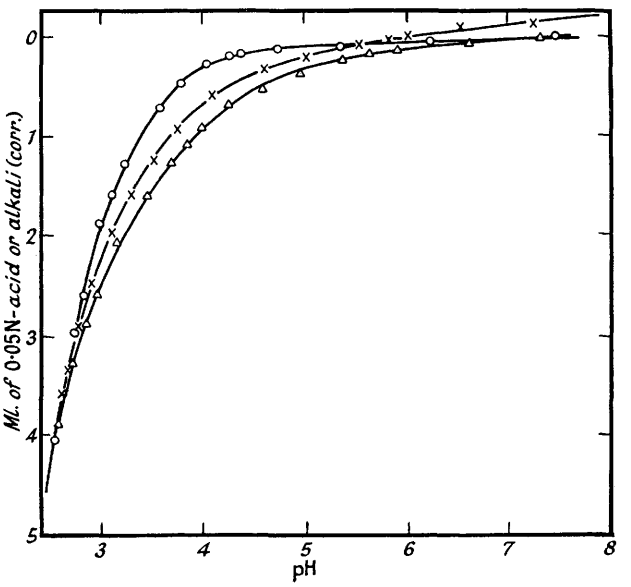

FIG. 8. *Titration curves of nucleic acid.*

○ *Forward-titration from* pH 7·5 *of untreated nucleic acid, and of nucleic acid treated with* 6M-*urea and dialysed.*

× *Back-titration of urea-treated nucleic acid.*

△ *Back-titration of untreated nucleic acid.*

molecular weights M, frictional coefficient f/f_0, and axial ratios ρ calculated from extrapolation of S and D to zero concentration. These are subject to considerable uncertainty for the reasons mentioned above, but they confirm the viscosity measurements by indicating a more compact molecule after treatment with urea and its subsequent removal.

Titration Curves.—As mentioned above, Gulland, Jordan, and Taylor (*loc. cit.*) regarded the hysteresis which they observed in the titration curves of the nucleic acid as evidence that hydrogen bonds were broken at extreme pH's. If phenol and urea break the hydrogen bonds the titration curves after such treatments should resemble the back-titration curve after treatment with acid or alkali. Experiments were made with the nucleic acid, after treatment with urea (6M) and removal of the urea by dialysis. On the alkaline side the results were vitiated by a small amount of urea remaining after dialysis (see below) which appeared to be hydrolysed to ammonium carbonate during the titration and gave rise to unsteady pH values. On the acid side no interference by urea was observed, and the forward- and back-titration curves of untreated and treated nucleic acid are shown in Fig. 8.

The amount of hysteresis observed in the urea-treated nucleic acid is appreciably less than that in the untreated material. This is similar to the effect of ultrasonic irradiation observed by Lee and Peacock (*J.*, 1951, 3374). However, the *forward*-titration curve is

identical in the two cases, which suggests that there is no effective difference in the titratable groups in the forward-titration, although the back-titrations differ somewhat. This must mean that the amino- and hydroxyl groups of the urea-treated nucleic acid are still inaccessible to titration by acid until fairly low pH's are reached; *i.e.*, if the original interpretation of the hysteresis is correct, they are still hydrogen-bonded in the urea-treated material. This indicates that the hydrogen bonds have been reconstituted although not necessarily between the same groups.

Effects of Small Concentrations of the Denaturing Agents.—If the effects of urea and phenol are denaturing processes involving the breakage of hydrogen bonds, they might be expected to occupy a finite time, as is usually the case in such observations as can be made with proteins. With the higher concentrations of urea and phenol, as used above, the viscosity is immediately affected by the addition, and then remains nearly constant. It was found, however, that with small concentrations of these agents, the initial change is small and is followed by a slow change of viscosity which leads eventually to values of the same order as those reached immediately in the presence of higher concentrations (Fig. 9).

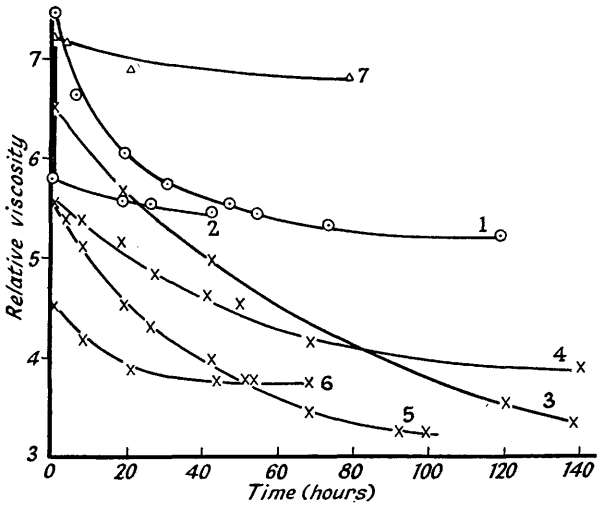

FIG. 9. *Effects of various concentrations of phenol and urea on 0·07% aq. sodium thymonucleate at* $\beta = 250$ *sec.*$^{-1}$.

1, 0·05M-*Phenol*. 2, 0·5M-*Phenol*.
3, 0·05M-*Urea*. 4, 0·15M-*Urea*.
5, 0·5M-*Urea*. 6, 6M-*Urea*.
7, *Control*.

EXPERIMENTAL

Materials.—Samples of deoxyribonucleic acid were prepared by the methods previously described (*J.*, 1950, 3411). Most of the present work was done with preparations from bovine thymus gland, but a few observations were made with a preparation from herring sperm which had similar sedimentation and viscosity constants. The preparations were all dialysed for a week or more with constant changes of distilled water and the acids were then precipitated with ethanol and dried.

Viscosity, Sedimentation, and Diffusion Measurements.—Viscosities were determined at various heads of solution in viscometers of the Frampton type. The mean shear rates β were calculated by Kroepolin's formula, $\beta = 8V/3\pi r^3 t$, where V is the volume (ml.) of liquid flowing in time t (sec.) through the capillary of radius r (cm.) (*Kolloid Z.*, 1929, **47**, 294).

The salt solutions were made up in the viscometer by inserting ground-glass weighing bottles containing the salt into a corresponding ground socket on the viscometer.

Sedimentation rates were determined with the Spinco ultra-centrifuge at approx. 60,000 r.p.m.

Diffusion rates were determined by observing the boundary between the solution and the corresponding solvent at different times by means of the schlieren optical system in a Perkin–Elmer electrophoresis apparatus.

Electrometric Titrations.—Aliquots (25 ml.) of 0·2% deoxyribonucleic acid solutions in 0·1N-potassium chloride, free from carbon dioxide, were titrated in a closed flask, with a glass

electrode which gave a linear pH response up to pH 13. The solutions were stirred by nitrogen throughout the titrations. In the acid direction the solutions were titrated with 0·05N-hydrochloric acid and in the alkaline direction with 0·05N-potassium hydroxide (carbonate-free). Corrections for water (Gulland, Jordan, and Taylor, *J.*, 1947, 1131) were determined by titrating an equal volume of 0·1N-potassium chloride from pH 7 to 2·5 and 11·5 respectively, under conditions similar to those used for the nucleic acid titrations.

In experiments with urea, recrystallized urea was added to the nucleic acid solutions to give a concentration of 6M. After 3 hours the mixture was dialysed in a rocking dialyser against a large volume of distilled water, which was changed six times. Air was excluded in this dialysis and all the water used had been previously boiled out and cooled in nitrogen.

Determination of Urea retained by the Nucleic Acid.—Since the urea produces irreversible effects it was desirable to determine the amount remaining after dialysis. The solution was depolymerized with deoxyribonuclease to release urea trapped in the polymerized form and was concentrated at 30° *in vacuo*. The urea present was then determined as ammonia by addition of urease, followed by potassium carbonate, in a Conway microdiffusion unit. A similar control experiment was made with a solution containing urea only. The concentration found in the nucleic acid solution was 4×10^{-4}M and that in the control 1×10^{-4}M. The difference is equivalent to 1 molecule of urea bound to every 20—25 nucleotide units. We are indebted to Miss E. M. Press for these analyses.

Solubility of Urea-treated Nucleic Acid.—During dialysis of nucleic acid in 6M-urea some material was always precipitated, but the amount varied considerably. The amount of the precipitate, determined by weighing after freeze-drying, was greater from a 0·4% than from a 0·1% nucleic acid solution, but the fraction of the whole precipitated was less in the former case. On one occasion, 15—20% of the nucleic acid was precipitated. When still wet in the solution the precipitates are soluble in 6M-urea, but after being dried are insoluble in both 6M-urea and 30% lithium bromide solution. On concentration of a 0·1% solution of the urea-treated nucleic acid (soluble part) to 0·4% at 35° *in vacuo*, no immediate precipitation occurred, but during 24 hours the solution deposited a gel-like precipitate on the walls of the vessel. When separated, this material is insoluble in water but is dispersed in 6M-urea to a fairly viscous opalescent solution.

When a dialysed solution of urea-treated nucleic acid is freeze-dried, the solid obtained is insoluble in water. It swells considerably but always remains as a separate phase.

These observations suggest that the precipitates obtained on removal of urea are aggregations formed by intermolecular formation of hydrogen bonds, which have been broken by the urea. The solubilization of the precipitates by urea will be due to disassociation of the aggregate by the further breakage of such bonds.

Effect of Salts on Nucleic Acid.—As lithium bromide has been stated to be effective as a hydrogen-bond breaker (Ambrose, Bamford, Elliott, and Hanby, *Nature*, 1951, **167**, 264), it was of interest to examine the effect of this and other salts. The normal effect of salts on the viscosity of nucleic acid solutions has been stated to be reversible (Greenstein and Jenrette, *loc. cit.*). A considerable measure of reversibility follows from the use of 2M-sodium chloride during the preparation. We have found, however, that complete recovery of the viscosity does not always occur after treatment with sodium chloride, followed by its removal by dialysis. The following table gives characteristic values. With sodium chloride a fairly good recovery is

Relative viscosities of nucleic acids.

Added salt :	NaCl (0·1N) *	LiBr (6N) †	NaCl (N) †	NaCl (0·1N) ‡
(1) In water	17·8	6·3	6·3	9·4
(2) In salt solution	3·3	1·7	2·1	4·4
(3) As (2), but dialysed ...	10·5	1·1	5·7	8·0

* Thymonucleic acid, 0·1%. † Thymonucleic acid, 0·07%. ‡ Herring sperm nucleic acid, 0·1%.

obtained with samples having a low initial viscosity, but not when the initial viscosity is high. With lithium bromide (6M) no recovery of the viscosity occurs. It appears from this that salts may bring about some permanent changes in the nucleic acid, but only in the case of the lithium salt does the effect approach that of urea and phenol. The great effect of salts on the viscosity is probably due to a decrease of the interactions between the nucleic acid particles. It is evident that, when interrupted, the interactions may not be fully restored by removal of the salt and that some change of configuration may occur. This is particularly marked with the more highly viscous samples of nucleic acid. This subject is being studied further.

Discussion

It has been shown that at high concentrations urea and phenol have a considerable effect on the viscosity of the nucleic acid and that this effect is irreversible in that the initial state is not restored when the added substance is removed. Detailed studies in the case of urea have shown that the effect observed in the presence of 6M-urea is similar to that produced by 0·1M-salts and is to be attributed for the most part to a decrease of the interaction of the particles, without it being necessary to postulate any great change of form of the particles themselves. However, when urea is removed by dialysis a permanent modification occurs, and observations of diffusion and sedimentation suggest that the particles assume a more compact form. Urea and phenol are well known to act as hydrogen-bond "breakers" and it is by virtue of this property that they denature proteins. It is probable that they do this by taking the place of either partner of the groups united by hydrogen bonds, which determine the configuration.

Urea molecules can no doubt form hydrogen bonds with each other, and in high concentrations it is likely that such associations occur. So long as a high concentration of urea is present, we can account for the absence of any significant change of configuration, although some of the configurational bonds may be broken, if the molecule is maintained in its original configuration by groups of urea molecules uniting the parts. When, however, the excess of urea is removed by dialysis, the particle of nucleic acid will be able to take up a new and more compact configuration. Phenol may be able to function similarly owing to the formation of complex molecules under the influence of the van der Waals attraction of the aromatic rings.

It seems unlikely that the amount of phenol or urea remaining after dialysis is sufficient to produce by itself a marked change of the behaviour of the nucleic acid. It would be sufficient to explain the observed effects, if the removal of the agent attached to some of the previously bonded amino- or hydroxyl groups permits rotation or bending of the particle, so that the latter takes up another configuration in which hydrogen bonds are re-formed, possibly with other partners.

That both urea and phenol when present in small concentrations produce similar effects, but at a very slow rate, also shows that the effect of these agents is not purely physical, such as might be produced by changing the environment of the nucleic acid particles (*e.g.*, by change of dielectric constant) but is due to a reaction which occurs at a finite rate. It is evident from the results that the final viscosities obtained with urea, at all the concentrations studied, are lower than those observed with *any* concentration of phenol. This suggests that urea brings about changes in the nucleic acid particles which are not effected by phenol, and this indicates that the hydrogen bonds concerned in these changes are not all of the same kind (cf. Cavalieri and Angelos, *J. Amer. Chem. Soc.*, 1950, **72**, 4686). Further experiments will be required to establish these conclusions fully but, since it is desirable to work at concentrations at which the nucleic acid particles do not interact with each other, it will be necessary to use experimental methods which are applicable at very low concentrations.

This work was communicated (in part) to the Protein and Nucleic Acid Conference held at New Hampton, N.H., in August 1951.

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