669. *The Action of Ionizing Radiations and of Radiomimetic Substances on Deoxyribonucleic Acid. Part I. The Action of Some Compounds of the "Mustard" Type.*

By J. A. V. Butler and K. A. Smith.

In the presence of di-(2-chloroethyl) sulphide ("mustard gas") and of di-(2-chloroethyl)-methylamine (I), deoxyribonucleic acid solutions, buffered at all pH's above 4, are degraded. They lose both their intrinsic viscosity and the additional apparent viscosity which is observed at low rates of flow. It is suggested that this action is related to the nucleotoxic effects of these and similar substances. 2-Chloroethyldimethylamine does not degrade the nucleic acid, but the analogous sulphur compound, 2-chloroethyl ethyl sulphide, does. Experiments with partly hydrolyzed (I) indicate that the molecular species which reacts with the nucleic acid is the same as that which combines with sodium thiosulphate.

It has been found that "mustard gas" [di-(2-chloroethyl) sulphide] and the analogous "nitrogen mustards," such as di-(2-chloroethyl)methylamine, produce biological effects similar to those produced by the action of *X*-rays and ultra-violet radiations on living tissues (see review by Boyland, *Ann. Review Biochem.*, 1949, **18**, 217). Among the effects which have been established with various compounds of this type are interference with mitosis and breakage of chromosomes (Darlington and Koller, *Heredity*, 1947, **1**, 187), induction of mutations (Auerbach, Robson, and Carr, *Science*, 1947, **106**, 243), and carcinogenesis (Boyland *et al.*, *Brit. J. Cancer*, 1948, **2**, 17).

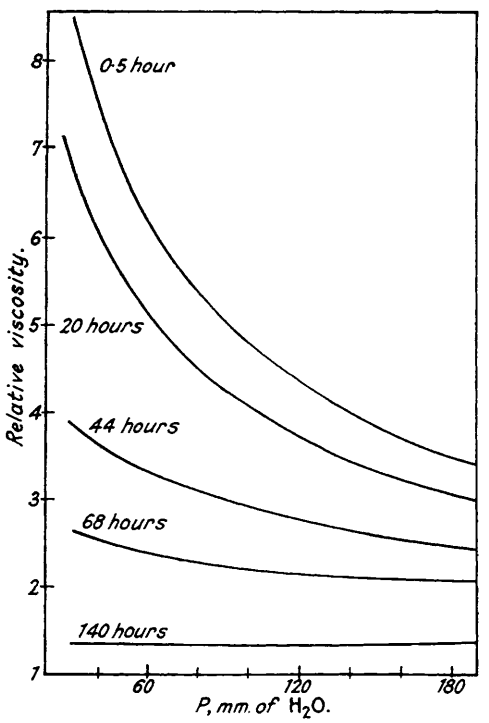
The action of *X*-rays and similar radiations on tissues and on isolated substances such as enzymes has been very extensively studied (see review by Dainton, *Ann. Reports*, 1948, **45**, 5; Dale, Gray, and Meredith, *Phil. Trans.*, 1949, **242**, 33) and it has been established that the

action is mainly indirect, being due to the primary products of the action of the radiations, *e.g.*, free hydrogen atoms and hydroxyl radicals, which then react chemically with the substances present in the solution, and *in vivo* with the constituents of the living cells.

It appeared to us that the origin of the effects on reproductive processes *in vivo* might be sought in the first place by examining the action of the substances, as well as of the radiations, on deoxyribonucleic acid, the characteristic nucleic acid of the nucleus and an important constituent of chromosomes. Some studies of this kind have already been made. Taylor, Greenstein, and Hollaender (*Cold Spring Harbor Symp.*, 1947, **12**, 237; *Arch. Biochem.*, 1948, **16**, 19) found thymus nucleic acid, a deoxypentose nucleic acid, to be degraded by *X*-rays. The structural viscosity (*i.e.*, the abnormally large component of the viscosity appearing at low rates of shear) is lost, the intrinsic viscosity at high rates of shear is diminished, and the loss of viscosity is accompanied by the loss of the original streaming birefringence. There is also a considerable change in the character and the speed of sedimentation in the ultra-centrifuge. These workers also found that much of the change of viscosity occurred as a slow change continuing for some time *after* irradiation.

FIG. 1.

Viscosity curves of 0·1% *thymus nucleic acid in* 0·1N-*sodium hydrogen carbonate at various times after addition of di-*(2-*chloroethyl) sulphide to* 0·0025M.



The action of some " nitrogen mustards " on deoxyribonucleic acid has been studied by Chanutin and Gjessing (*Cancer Research*, 1946, **6**, 593). Although they observed a decrease in viscosity in the presence of di-(2-chloroethyl)-methylamine and tri-(2-chloroethyl)amine, their observations did not permit distinguishing the structural component of the viscosity observable at low rates of shear; further, under their experimental conditions (*e.g.*, period of observation) the changes observed in the nucleic acid were comparatively small.

In a study of the action of di-(2-chloroethyl) sulphide on deoxyribonucleic acid in 35% aqueous alcohol, Elmore, Gulland, *et al.* (*Biochem. J.*, 1948, **42**, 308) isolated two products of which one gave more viscous solutions than the original nucleic acid.

We have examined the action of four compounds of the " mustard " type on the nucleic acid in sodium hydrogen carbonate solution, and in the case of di-(2-chloroethyl)methylamine in a variety of buffered solutions. Both di-(2-chloroethyl) sulphide and the typical " nitrogen mustard " di-(2-chloroethyl)methylamine greatly diminish the structural and the intrinsic viscosities, the final product being a Newtonian liquid with a viscosity not much exceeding that of water.

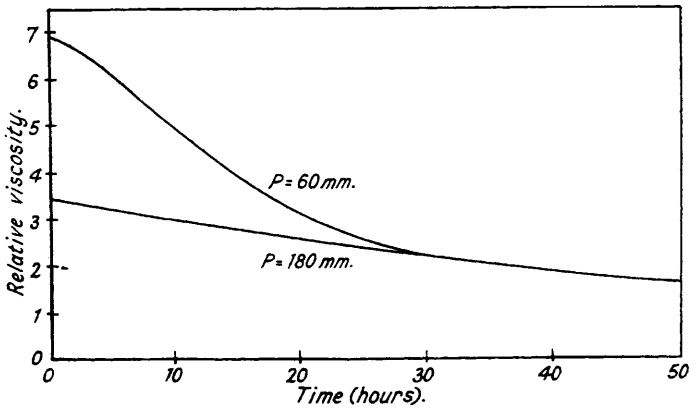
Fig. 1 shows the viscosity curves determined at various times during the reaction of di-(2-chloroethyl) sulphide in 0·1N-sodium hydrogen carbonate. The substance was added, dissolved in a little alcohol. It was ascertained that the alcohol alone did not affect the viscosity appreciably. The action of di-(2-chloroethyl)methylamine is very similar. On the other hand, 2-chloroethyldimethylamine had no appreciable effect on the viscosity over a long period. The analogous sulphur compound, 2-chloroethyl ethyl sulphide, was however about one-fifth as effective as di-(2-chloroethyl) sulphide. 2-Chloroethyl 2-hydroxyethyl sulphide was fairly effective as a degrading agent, but the substance used may have been contaminated with the dichloro-compound; to account for the effect observed it would be necessary to assume that the sample used contained at least 20% of the dichloro-compound.

Fig. 2 shows the change of viscosity with time in a typical instance. Two curves are shown, representing the low- and the high-pressure viscosities respectively. For the former we have arbitrarily taken the viscosity at a pressure equivalent to a head of 60 mm. of water. The latter, at a pressure of 180 mm. of water, begins to approach the intrinsic viscosity of the

solution, and no great error is made in regarding it as such. The fact that the two curves coalesce after some time shows that the " structural " component of the viscosity is lost while the intrinsic viscosity continues to change.

FIG. 2.

Change of viscosities with time for 0·1% *thymus nucleic acid in* 0·1N-*sodium hydrogen carbonate after addition of* 0·004 *g.-mol. of di-(2-chloroethyl)methylamine per l., as measured in the Frampton viscometer.*



Similar effects are observed in solutions buffered at pH's down to about 4·0. Above this the effect of pH on the rate of change of viscosity is evidently not very great, but it is not easy to deduce with any precision the effect of the pH alone. For example, in phosphate buffers the rate diminishes with increasing phosphate concentration owing, no doubt, to the competition of the phosphate ions for the reactant molecules. Very low buffer-ion concentrations are not desirable, as a fair concentration of buffer salts is necessary to prevent appreciable pH changes during the reaction. As an approximate measure of the reaction rate with nucleic acid we have taken the time required for the loss of half of the low-pressure viscosity. These reaction times for a variety of solutions are given in the Table; they represent only the approximate relative rates of the action for a given sample of the nucleic acid. We have mostly used two preparations of the nucleic acid, of which the second B was less highly polymerized than A, as shown by their viscosities. As might have been expected the initial rate of degradation

Relative rates of degradation of deoxyribonucleic acid.

Compound.*	Concn., M.	Nucleic acid, sample.	Nucleic acid, concn., %.	Buffer salts.	pH.	Hrs. for half-change.
I	0·0004	A	0·1	0·1N-$NaHCO_3$		*ca.* 80
I	0·0020	A	0·1	,,		13
I	0·0042	B	0·1	,,	9·3—9·1	14
I	0·0084	B	0·1	,,		8
I	0·0084	B	0·2	,,		8
I	0·008	B	0·2	0·05M-Borate	8·5—3·0	4·5
I	0·0086	B	0·2	0·1M- ,,	8·5—8·4	15
I	0·0090	B	0·2	0·028M-Phosphate	7·2—6·28	8
I	0·0092	B	0·2	0·1M- ,,	7·3—6·8	32
I	0·002	A	0·1	0·045M- ,,	7·0—6·8	20
I	0·0088	B	0·2	0·1M-Acetate	5·6—5·46	12·5
I	0·0087	B	0·2	0·2M- ,,	4·5—4·35	*ca.* 30
I	0·0084	B	0·2	0·1M-Citrate	4·0—3·9	little change in 72
II	0·006	A	0·1	0·1N-$NaHCO_3$		no change
I + II	I, 0·002; II, 0·006	A	0·1	,,		26
III	0·025	A	0·1	,,	9·3—9·1	*ca.* 20
IV	0·0025	A	0·1	,,		40
I	0·0084	C	0·14	,,		8·5
I	0·008	S†	0·09	,,		7·0

* I, Di-(2-chloroethyl)methylamine. II, 2-Chloroethyldimethylamine. III, Chloroethyl ethyl sulphide. IV, Di-(2-chloroethyl) sulphide.

† A sample prepared by Prof. R. Signer.

of A was greater than that of B. It appears then that the more highly polymerised acid is initially more rapidly degraded, and this affects the times required for the loss of half the viscosity. Apart from this the general course of the reaction was similar in the two cases.

It can be seen that under comparable conditions the rate is proportional to the concentration of the reagent. The time of half-change also appears to be independent of the concentration of the nucleic acid within the rather narrow limits investigated. It is uncertain whether 2-chloroethyldimethylamine reacts with nucleic acid or not, although it produces no change of viscosity. This cannot be readily discovered by analysis of the product. In order to see if its presence affected the behaviour of di-(2-chloroethyl)methylamine, the effect of both together was examined. The change of viscosity was considerably slower than that produced by the same concentration of di-(2-chloroethyl)methylamine alone. This might mean that the monochloro-compound, by combining with the nucleic acid at some reactive points, hindered attack by the dichloro-amine; but it is also possible that they react with each other. It is well known (Hanby and Rydon, *J.*, 1947, **513**, 519) that di-(2-chloroethyl)-methylamine readily dimerizes; it also reacts with primary amines (Ross, *J.*, 1949, 2824); so it may react with 2-chloroethyldimethylamine, which will then be a competitor of the nucleic acid.

It has been pointed out (Ogston *et al.*, *Trans. Faraday Soc.*, 1948, **44**, 45; Ross, *J.*, 1950, 815) that sulphur compounds with an unattached electron pair react very readily with compounds of the type being considered. We found that in the presence of an equimolecular quantity of cysteine the change normally produced by di-(2-chloroethyl)methylamine is almost completely inhibited.

It has been suggested (Goldacre, Loveless, and Ross, *Nature*, 1949, **163**, 667; Loveless and Revell, *ibid.*, p. 938) that the biological activity of the " two-handed " (dichloro) " mustards " is connected with their ability to form a cross-link between two elements of the chromosome threads, or possibly some other structures, in the mitotic cell. The observations described above give no support to this theory, unless the initial small rise of viscosity sometimes observed indicates an initial cross-linking reaction, which is followed by a breakdown of the cross-linked chains. The occurrence of cross-links, however, is supported by the observation of Elmore, Gulland, *et al.* (*loc. cit.*) that in their " more viscous " product two phosphoryl groups are blocked for each " mustard " molecule reacting. The conditions of their experiments (concentrated solutions in 35% alcohol) were, however, very different from those in ours. As cross-linking between two nucleic acid chains will occur more readily the closer together are the chains, *i.e.*, in solutions more concentrated with respect to nucleic acid, an attempt was made to produce more favourable conditions by adding more concentrated nucleic acid to a concentrated buffer solution containing the reagent. After a period in which the reaction was expected to be complete, the solution so obtained was diluted to the same concentration as in the previous experiments. No significant difference in the final viscosity was observed, even when the reaction began with the nucleic acid present in the solid state. In fact, the degradation occurred rather more rapidly in the concentrated solution, as might be expected from the increase of concentration of the reactants.

In practically every case the final result was a solution with relative viscosity between 1 and 2. The only exception was in the experiment in which the concentration of di-(2-chloroethyl)methylamine was 0·0004M. and the time of half-change was of the order of 80 hours; in this time the degradation had become very slow. With this exception, it appears that at the concentrations employed, there was sufficient reagent present to bring about a complete loss of the characteristic viscosity of the nucleic acid. It will be shown in later papers that the average molecular weight of the products is of the order of 10^5. If the original molecular weight is taken to be of the order of 10^6, the original molecular concentration in a 0·1% solution of the nucleic acid is of the order of 10^{-6}M. and that in the final degraded solutions 10^{-5}M. It is obvious that in the solutions used the concentrations of the reagents (10^{-3}—10^{-2}M.) are much greater than the number of fragments formed, so that there would appear to be sufficient reactant to produce a complete degradation even if a considerable number of molecules are required to produce each break of the nucleic acid molecule.

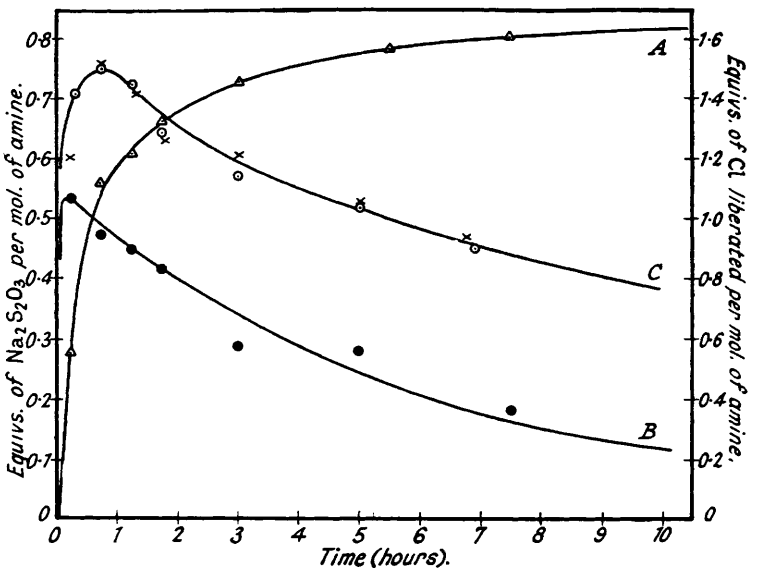
Experimental.

Preparation of the Sodium Salt of Deoxypentose Nucleic Acid.—The method described by Gulland, Jordan, and Threlfass for the isolation of the sodium salt of the deoxypentose nucleic acid of calf thymus was employed with a few minor modifications. Separation of the emulsion layer during the removal of the protein from the nucleo-protein by shaking with chloroform–amyl alcohol was effected by centri-

fugation on an International Centrifuge at 2000 r.p.m. After 8—9 emulsifications no stable gel was formed. The nucleic acid, after precipitation with alcohol as described by the above authors, readily dissolved in water to give a 0·25% solution. The very faintly turbid solution was clarified by centrifugation at 30,000 r.p.m. in the closed bowl of the Sharples supercentrifuge for 60 minutes or for 60 minutes at 50,000 r.p.m. by using the preparative rotor of the Spinco ultracentrifuge. Traces of solid material separated which gave positive tests for protein, and the supernatant liquid was clear. The aqueous solution of the nucleic acid was dialysed against distilled water and finally the substance was dried from the frozen state. Analytical figures varied slightly from batch to batch, but the nitrogen : phosphorus ratio was constant. Nitrogen was determined by the micro-Kjeldahl method, and phosphorus colorimetrically (Found, Prep. A : N, 13·6; P, 8·0. Prep. B : N, 15·2; P, 9·0. Prep. C : N, 14·8; P, 8·8%).

FIG. 3.

Liberation of Cl^-, *and sodum thiosulphate titre, of solutions of di-(2-chloroethyl)methylamine in sodium hydrogen carbonate solutions.*



A.—Liberation of Cl^- *under conditions of prehydrolysis in* N*-sodium hydrogen carbonate (amine =* 0·08M.). *B.—Thiosulphate titre of the amine during prehydrolysis in* N*-sodium hydrogen carbonate (amine =* 0·08M.). C_0*—Thiosulphate titre of the amine* (0·008M.) *during hydrolysis in* 0·1N*-sodium hydrogen carbonate.*

—O—O—O— *Without nucleic acid.* —×—×—×— *In presence of nucleic acid.*

Viscosity Measurement.—The viscosity was measured in viscometers of the Frampton type (*J. Biol. Chem.*, **129**, 233, 1939) consisting of two wide, cylindrical tubes of uniform bore, joined by a horizontal capillary. The times of flow between a series of marks at measured distances on one of the wide tubes were determined. If Δt is the time of flow between two marks at which the distances between the meniscus levels in the two limbs are h_1 and h_2, the relative viscosity is proportional to $\gamma = \Delta t/\log(h_1/h_2)$. The viscometer is calibrated with water, which gives a constant γ_w at all levels. The relative viscosity γ/γ_w is plotted in Fig. 1 against the mean pressure difference at which the reading is taken. All measurements were at 25°.

Action of Partly Hydrolysed Solutions of Di-(2-chloroethyl)methylamine on the Nucleic Acid.—To get information about the course of the reaction, so far as it is affected by changes in the degree of hydrolysis of di-(2-chloroethyl)methylamine, we have examined the effect of solutions of this amine partly hydrolysed in N-sodium hydrogen carbonate before addition to the nucleic acid. Fig. 3 shows the changes which occur during the prehydrolysis. *A* represents the liberation of Cl^-, and *B* the amount of sodium thiosulphate used in 10 minutes when added to the solution diluted to 0·1N. with respect to sodium hydrogen carbonate. It can be seen that the first chlorine atom is completely ionized in 30—45 minutes, while the second is liberated much more slowly. The thiosulphate titre, which may be taken to represent the concentration of the reactive molecules, is a maximum near the beginning and decreases to zero in about 16—20 hours. Fig. 3 also shows the thiosulphate titre (curve *C*) in 0·1N-sodium hydrogen carbonate, with and without nucleic acid. This nucleic acid solution was that used in the viscosity experiments. It can be seen that the presence of nucleic acid has very little effect on this titre, indicating that the nucleic acid is not directly involved in the rate-determining process.

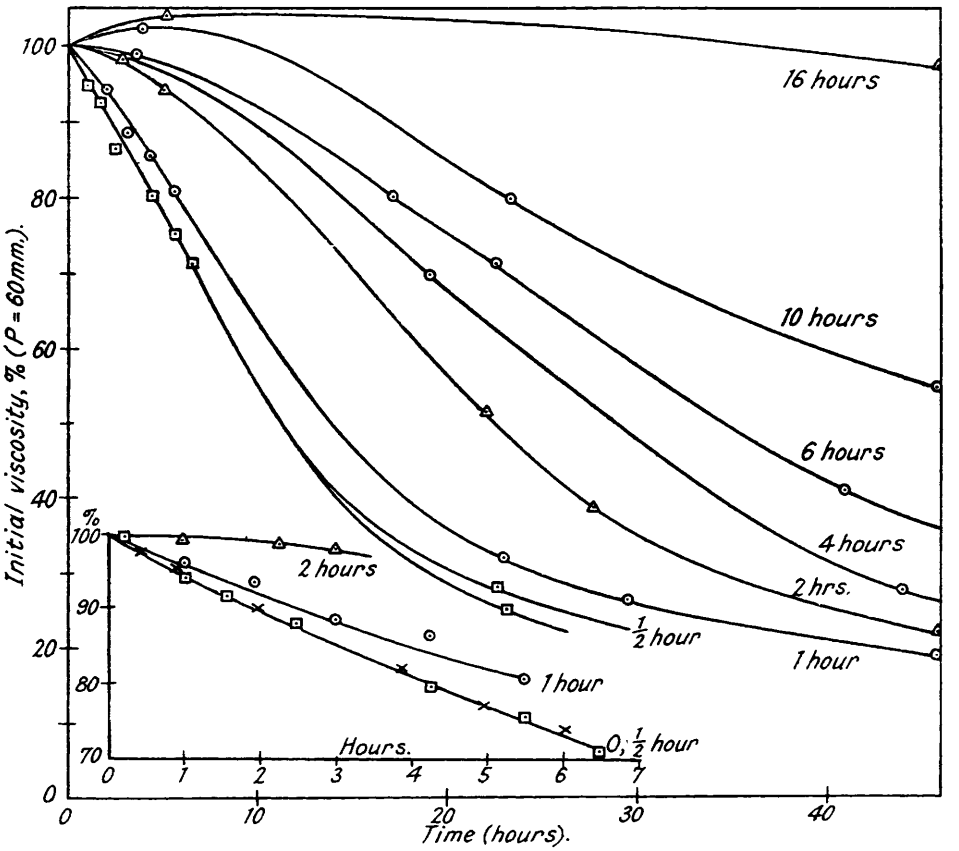
The changes of viscosity of a nucleic acid solution when di-(2-chloroethyl)methylamine, prehydrolysed for various times in N-sodium hydrogen carbonate, is added (so that the final solution is 0·1N-$NaHCO_3$)

are shown in Fig. 4. It can be seen that for the first hour the prehydrolysis had very little effect on the rate of loss of viscosity; but with longer prehydrolysis the reaction took on a different character, with a well-marked induction period. After about 16 hours' prehydrolysis the solution lost the ability to bring about the change in viscosity.

Different behaviours in the *initial* stages of the reaction have been observed with different samples of nucleic acid. One sample (B), when treated in the usual way with di-(2-chloroethyl)methylamine in 0·1N-sodium hydrogen carbonate, exhibited a small initial rise of viscosity during the first hour, which then gave place to the usual loss of viscosity. When this sample was treated with partly hydrolysed solutions of the amine, the initial rise was greatly increased but the subsequent course of the reaction

FIG. 4.

Action of 0·14% thymus nucleic acid solutions of di-(2-chloroethyl)methylamine (0·008M.) prehydrolysed for various times in N-sodium hydrogen carbonate.



Prehydrolysis times are marked on individual curves; abscissæ refer to the degradation of the nucleic acid as measured by the percentage of the initial viscosity.

was not affected, the times required for the loss of the viscosity being about the same. The initial rise appears to be caused by the action on the nucleic acid of a product of hydrolysis of the amine. This is confirmed by the fact that di-(2-hydroxyethyl)methylamine gave rise, with this nucleic acid (with 0·1N-sodium hydrogen carbonate), to a considerable increase in viscosity (50% in 6 hours). A smaller effect was observed with the other samples of nucleic acid employed. The most probable cause of the apparent induction period observed in Fig. 4 is that a product of the hydrolysis of the amine, not necessarily the dihydroxy-amine, causes a slow increase in viscosity in the initial stages of the reactions which is superimposed on the normal decrease brought about by the reagent. The effect of this is not usually apparent with the rapidly decreasing viscosities produced by the dichloro-amine; but when superimposed on the more slowly decreasing viscosities produced by the partly hydrolysed solutions, it may give rise to a stationary or in some cases an increasing viscosity.

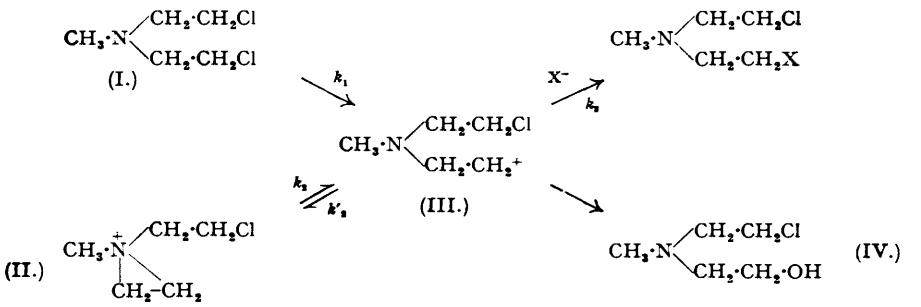
The cause of the rise of viscosity produced by certain substances on some samples of nucleic acid requires further investigation. It may perhaps be caused by an adjustment of the state of the nucleic acid to the presence of a new solute, which interacts with it in some way. The main conclusions of

this paper as to the effect of the " mustards " on the nucleic acid depend on the complete loss of the characteristic viscous behaviour of the nucleic acid and are not affected by this phenomenon.

Discussion.

It is difficult to deal with the kinetics of these effects with any preciseness, since it is not known what reactions underlie the observed changes of viscosity or how the viscosity is related to the state of the nucleic acid. The change of viscosity with time is often not far from linear over the greater part of its course, while the concentration of the reactive substance measured by the thiosulphate titre diminishes rapidly. Some rather general conclusions about the nature of the reaction can however be drawn.

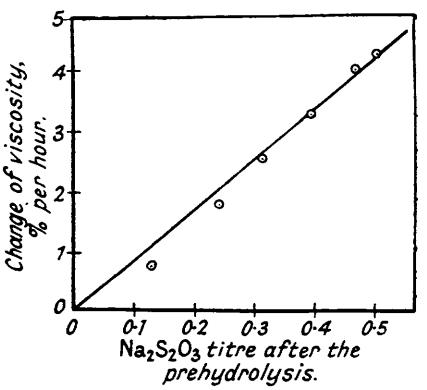
The following scheme of the initial stages of the hydrolysis of di-(2-chloroethyl)methylamine has been established, in its essentials (Hanby and Rydon, *J.*, 1947, **513**, 519; Golumbic, Fruton, and Bergmann, *J. Org. Chem.*, 1946, **2**, 518 *et seq.*; Stein, Moore, and Bergmann, *ibid.*, p. 664; Ross, *J.*, 1949, 183, 1977, 2589, 2824; 1950, 815; Bartlett, Ross, and Stein, *J. Amer. Chem. Soc.*, 1947, **64**, 2971, 2977; 1949, **71**, 1406, 1415; Cohen, Artsdalin, and Harris, *ibid.*,



1948, **70**, 281). In this, X^- represents an ion, such as that of nucleic acid, with which the substance reacts. It should be noted that various other possibilities such as dimerization reactions, reactions with the inorganic anions present, or multiple reactions with nucleic acid, may be complicating factors. The actual reactive agent present is generally believed to be the carbonium ion (III), or more stable forms such as the cyclic compound (II), or the equivalent open form, stabilized by resonance (cf. Butler, *Nature*, 1950, **166**, 18).

Fig. 5.

Rate of change of viscosity, and thiosulphate titre, of the prehydrolysed solutions.



The fact that the initial rate of degradation is practically independent of the amount of the first chlorine atom which has been ionized shows that both di-(2-chloroethyl)methylamine (I) and its first ionization product are equally effective. This will be so if $k_2 > k_1$, $k_3 > k_1$, and k_1 and k'_2 are of the same order of magnitude. The effect of the first ionization in these circumstances is to provide a supply of a comparatively stable intermediate, such as (II), from which the reactive agent (III) can be regenerated. The rate of the reaction will be determined by the rate at which (III) is formed and will thus be determined by $k_1[\mathrm{I}] + k_2[\mathrm{II}]$ and will be independent of the degree of ionization so long as $([\mathrm{I}] + [\mathrm{II}])$ is approximately constant and $k_1 = k'_2$.

With longer periods of prehydrolysis the initial amount of (II) will gradually diminish and the rate of formation of (III) will be correspondingly less. If the thiosulphate titre is taken as a measure of the amount of (II), the initial rate of loss of viscosity should be proportional to it. As mentioned above, however, the change of viscosity in the early stages of the reaction is complicated by an extraneous phenomenon and we are unable to use the initial change of viscosity with time as a measure of the initial rate of the reaction. Since however the viscosity curve is not far from linear in those cases where it is uncomplicated, there will probably be no very great error in taking the rate of change of viscosity, when half the original viscosity has been lost, as representative of the true rate of change near the start of the action. This has

been done in Fig. 5, which shows that the rate of change of viscosity, as corrected in this way, is roughly proportional to the thiosulphate titre of the prehydrolysed solution. It is thus reasonable to conclude that the molecular species bringing about the change of viscosity of the nucleic acid is the same as that which reacts with thiosulphate.

Finally, these experiments do not indicate clearly if the chloro-alcohol (IV) is capable, after ionization, of degrading nucleic acid. It is obvious that some chloro-alcohol must be present during all but the early stages of the reaction. On the other hand in the prehydrolysis experiments, complete loss of the ability to degrade nucleic acid occurs while 30% of the second chlorine atom is still un-ionized. This however is not conclusive as this chlorine atom ionizes very slowly and is probably present in a dimeric product rather than in the simple chloro-alcohol.

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