113. *The Action of Ionizing Radiations and of Radiomimetic Substances on Deoxyribonucleic Acid. Part IV.* The Products of the Action of Di-(2-chloroethyl)methylamine.†*

By E. M. Press and J. A. V. Butler.

An examination has been made of the products formed when di-(2-chloroethyl)methylamine acts in dilute sodium hydrogen carbonate solutions on deoxyribonucleic acid of calf thymus. The main analytical difference between the product and the original nucleic acid is a decrease in the amount of primary amino-nitrogen and in that of purine nitrogen, precipitable as purine silver salt after hydrolysis with dilute sulphuric acid. The latter is appreciably greater than the total decrease of primary amino-nitrogen. Experiments have also been made on the action of the base on certain ribonucleosides.

It has been shown (Part I, *J.*, 1950, 3411) that both di-(2-chloroethyl)methylamine and di-2-chloroethyl sulphide cause a " degradation " of the deoxyribonucleic acid of calf thymus in aqueous solutions of pH > 4, which is shown by the almost complete loss of the characteristic viscosity which occurs. In the case of the amine, it has been shown (Part III, *J.*, 1950, 3421) that the loss of viscosity is accompanied by a decrease of the apparent molecular weight. A knowledge of the chemical changes producing these physical effects would be of great interest, in view of the powerful biological actions of these and similar substances (see Part I for references) and the indications that deoxyribonucleic acids are particularly sensitive to them (Herriott, *J. Gen. Physiol.*, 1948, **32**, 221). The action of di-2-chloroethyl sulphide on thymonucleic acid was studied by Elmore, Gulland, Jordan, and Taylor (*Biochem. J.*, 1948, **42**, 308), who isolated two products of the reaction and from their titration curves concluded that the sulphur " mustard " had reacted with (1) the primary phosphoryl groups, (2) the purine-pyrimidine hydroxyl groups, together with (3) an uncertain amount of amino-groups. In order to interpret the physical effects reported in previous papers, and in view of the rather different conditions of the experiment of Elmore *et al.*, we have made a partial chemical examination of the products of the action of the amine on the nucleic acid in dilute sodium hydrogen carbonate. To assist this interpretation we have also made experiments on the action of di-(2-chloroethyl)-methylamine on some ribonucleosides.

Experimental

Preparation of the Thymonucleic Acid.—The preparations B and G/2 were prepared as described in Part I (*loc. cit.*). They were similar in physical properties and their analyses are given in Table 2. S/1 was a preparation by H. Schwander (Prep. VII) kindly given to us by Professor R. Signer.

* Part III, *J.*, 1950, 3421.

† Read, in abstract, at the XIIth International Congress of Pure and Applied Chemistry, New York, September 1951.

Preparation of the Reaction Product of Nucleic Acid with Di-(2-chloroethyl)methylamine.—*Preparation B/1.* 250 Mg. of the base were added to 200 mg. of nucleic acid in 200 ml. of 0·1N-sodium hydrogen carbonate saturated with chloroform in three portions during 72 hours at room temperature. After 24 hours more, the solution was dialysed for 6 days against frequent changes of distilled water at 2°. The dialysate was neutralised with acetic acid and concentrated to 25 ml. *in vacuo*. The residue was freeze-dried.

Preparation G/2/1. This was similarly prepared by adding, during 5 days, 600 mg. of base to 500 mg. of nucleic acid in 250 ml. of 0·1N-sodium hydrogen carbonate.

Preparation S/1/1 was similarly prepared by adding in two portions 300 mg. of base to 200 mg. of S/1 in 200 ml. of 0·1N-sodium hydrogen carbonate, followed by dialysis and freeze-drying.

Analytical.—Total nitrogen was determined by the micro-Kjeldahl method, and phosphorus colorimetrically by a modification of Martland and Robison's method (*Biochem. J.*, 1926, **20**, 847). Purine-nitrogen was determined by precipitation of the bases with saturated silver sulphate in dilute sulphuric acid at 2° (see Gulland, Jordan, and Threlfall, *J.*, 1947, 1129). Conditions of hydrolysis of the nucleic acid and time of digestion were varied as shown in the table. Finally, the second method of hydrolysis and digestion overnight were adopted for all determinations.

Results.

	Time of digestion, minutes	Purine-N, %	Pyrimidine-N, %	Recovery of total N, %
Hydrolysis 1. Solution of thymonucleic acid in 5·0% H_2SO_4 refluxed at 100° for 2 hours	60	8·7	5·7	95
	75	8·7	5·6	94
	90	9·05	6·0	99
	Overnight	9·05	5·9	98
Hydrolysis 2. Solution of thymonucleic acid in N-H_2SO_4 at 100° for 1 hour	40	7·7	5·6	89
	60	8·8	5·6	97
	90	9·0	6·0	99
	Overnight	9·0	6·0	99

The following results, obtained after hydrolysis of a mixture of purine and pyrimidine nucleosides, show that the method gives a quantitative precipitation of the purines under the conditions used :

	Mg. of N per ml. determined alone	Mg. of purine-N determined in mixture of 1 ml. of each	Mg. of pyrimidine-N determined in mixture of 1 ml. of each
Guanosine	0·195		
Adenosine	0·251	0·450	—
Cytidine	0·148	—	0·268
Thymine	0·115	—	

From a comparison of the results obtained with thymus deoxyribonucleic acid (Table 2) with those obtained recently with similar material, chromatographic methods being used to estimate the separate purines, it would appear that the silver method gives rather low values for the purines in the hydrolysis products of the natural nucleic acid (*e.g.*, Marshak and Vogel, *J. Biol. Chem.*, 1951, **189**, 597; Chargaff, Vischer, Doniger, Green, and Misani, *ibid.*, 1949, **177**, 405; Wyatt, *Biochem. J.*, 1951, **48**, 584). The values obtained by these workers for the ratio of purine-nitrogen to -phosphorus are in the region of 2·4—2·5 but the range of values is rather wide. Using the alternative copper hydrogen sulphite method of purine precipitation (Kerr and Blish, *J. Biol. Chem.*, 1932, **98**, 193; Kerr, *ibid.*, 1940, **132**, 147), we obtained the following results which are appreciably higher than those obtained by the silver method with the same preparation and higher than those reported by other authors (*loc. cit.*) :

	Purine N/P (± 0·09)		Purine N/P (± 0·09)
Nucleic acid B	2·68	Nucleic acid (preptn. B/1) treated with base	2·42

but a similar decrease of the precipitable purine after treatment with di-(2-chloroethyl)methylamine is observed.

It is concluded that, although the purine nitrogen/phosphorus ratio determined by the silver method may be somewhat low, the relative values obtained with the nucleic acids and their reaction products are significant.

Solubility of Silver Salts of Alkylated Purines.—The silver method of purine analysis depends on the fact that both guanine and adenine are quantitatively precipitated as silver salts in dilute

sulphuric acid. In order to find the effect of various substituents of the purine nucleus on the precipitability under these conditions a number of alkylated derivatives have been examined, with the results shown in Table 1. Results obtained with the simple purines are included to show the concentrations employed. The nucleic acid solutions were determined at a concentration equivalent to $7{\cdot}0 \times 10^{-4}$M with respect to purines.

It can be seen from this that methylation of guanine in the 1- or the 7-position does not interfere with the formation of an insoluble silver salt; in the xanthine series methylation in the 3-, the 3 : 7-, and the 1 : 3 : 7-positions and also in the 1 : 9- and the 1 : 3 : 9-positions prevents the formation of an insoluble silver salt, but with 1-, 7-, and 9-methyl- and 1 : 3- and 1 : 7-dimethyl-xanthine no precipitate is formed at the concentration normally used but may be formed at a higher concentration.

TABLE 1. *Precipitation of purine derivatives by saturated silver sulphate in dilute sulphuric acid.*

Base	Concn. (M)	H_2SO_4 concn. (M)	Recovery of N, %	Remarks
Guanine	$7{\cdot}0 \times 10^{-4}$	0·1—0·7	100	—
Adenine	$7{\cdot}0 \times 10^{-4}$	0·25—0·5	96	—
Hypoxanthine	$1{\cdot}0 \times 10^{-3}$	0·5	98	—
1-Methylguanine	$1{\cdot}2 \times 10^{-3}$	0·3	99	Ppt. did not dissolve when diluted 2-fold with N-H_2SO_4
7-Methylguanine	$1{\cdot}1 \times 10^{-3}$	0·3	98	As above
1 : 7-Dimethylguanine	$1{\cdot}0 \times 10^{-3}$	0·3	85	As above. This compound is possibly impure
1-Methylxanthine	$1{\cdot}6 \times 10^{-3}$ (sat.	0·5	—	No ppt. at room temp.; ppt. formed in 1 hr. at 2° which dissolves on 4-fold dilution
1 : 3-Dimethylxanthine	$1{\cdot}7 \times 10^{-3}$	0·3	—	No ppt. at 2°
,,	$1{\cdot}4 \times 10^{-2}$	0·5	—	Ppt. at 2° which dissolves on 2-fold dilution
3 : 7-Dimethylxanthine	$4{\cdot}3 \times 10^{-3}$ (sat.)	0·5	—	No ppt. after 24 hr. at 2°
1 : 3 : 7-Trimethylxanthine	$3{\cdot}3 \times 10^{-2}$	0·5	—	,, ,,
3-Methylxanthine	$7{\cdot}7 \times 10^{-4}$ (sat.)	0·3	—	No ppt. after 48 hr. at 2°
7-Methylxanthine	$2{\cdot}4 \times 10^{-3}$	0·3	79	No ppt. at room temp., ppt. at 2°
	$1{\cdot}8 \times 10^{-3}$	0·3	46	
9-Methylxanthine	$2{\cdot}2 \times 10^{-3}$	0·3	58	,, ,, ,,
	$1{\cdot}1 \times 10^{-3}$	0·5	—	No ppt. at 2°
1 : 7-Dimethylxanthine	$2{\cdot}3 \times 10^{-3}$	0·3	32	No ppt. room temp.; ppt. at 2°
1 : 9-Dimethylxanthine	$3{\cdot}9 \times 10^{-3}$	0·5	—	No ppt. after 48 hr. at 2°
1 : 3 : 9-Trimethylxanthine	$2{\cdot}0 \times 10^{-3}$	0·3	—	No ppt. after 48 hr. at 2°
Xanthosine	$1{\cdot}3 \times 10^{-3}$	0·3	—	,, ,,

Estimation of Deoxypentose by Diphenylamine Reaction.—Seibert's modification (*J. Biol. Chem.*, 1940, **133**, 594) of the original Dische reaction (*Mikrochemie*, 1930, **8**, 4) was employed.

Estimation of Free Amino-groups.—Van Slyke's method was used (*J. Biol. Chem.*, 1912, **12**, 275) with the reaction vessel due to Koch (*ibid.*, 1929, **84**, 601). For the nucleosides a reaction time of 70 minutes was found to be satisfactory. Nucleic acid was hydrolysed for 1 hour in N-sulphuric acid at 100° before estimation, and for reproducible results a reaction time of 2 hours was necessary.

Reaction of Di-(2-chloroethyl)methylamine with Nucleosides.—In view of the apparent loss of purine-nitrogen in the reaction with the nucleic acid, we have examined the reaction of the base with the purine nucleosides guanosine and adenosine and also with the pyrimidine nucleoside cytidine, under conditions similar to those employed with nucleic acid. These experiments were carried out with ribonucleosides derived from yeast, kindly given to us by Dr. R. J. C. Harris, since no deoxyribonucleosides were available.

Experiment I. Approx. 0·05% solutions of guanosine or adenosine in 0·1M-sodium hydrogen carbonate were kept for 4 days with the amine (12 mols. to 1 mol. of nucleoside) at room temperature, and the solution was made 1N with respect to sulphuric acid and heated for 20 minutes at 100°. Purine-nitrogen was then determined as described above. Control solutions of the nucleosides in sodium hydrogen carbonate were kept at room temperature for the same period, and the purine-nitrogen similarly estimated. The results are given in Table 3. In control experiments a 100% recovery of purine was obtained in the presence of the hydrolysis products of the amine. No purine was precipitated with silver sulphate when the treated nucleoside was not hydrolysed with sulphuric acid, and thus no free purine was liberated by the reaction of the nucleoside with the di-(2-chloroethyl)methylamine.

Experiment II. Adenosine.

A. 56·3 Mg in 5·5 ml. of 0·5M-sodium hydrogen carbonate were made up to 0·8M with sodium hydroxide and left at room temperature for 4 days.

A1. 2·5 Ml. of solution A were made up to 5 ml. with glacial acetic acid, and 2-ml. samples were used for estimation of primary amino-nitrogen. 0·2 Ml. was hydrolysed in N-sulphuric acid and purine-nitrogen estimated with silver sulphate.

A2. 2·5 Ml. of A were neutralised and brought up to N with concentrated sulphuric acid, heated for 1 hour at 100°, and made up to 5 ml. with water. 2 Ml. of this solution were used for determination of primary amino-nitrogen and 0·2 ml. for determination of purine-nitrogen.

B. 61·4 Mg. in 5·5 ml. of 0·5M-sodium hydrogen carbonate were made up to 0·8M with sodium hydroxide. 500 Mg. of amine were added, and the whole was left at room temperature for 4 days.

B1. 2·5 Ml. of solution B were made up to 5 ml. with glacial acetic acid and estimated as for A1.

B2. 2·5 Ml. of B were neutralised and made N with concentrated sulphuric acid, heated for 1 hour at 100°, and made up to 5 ml. with water; estimation was as for A2.

Cytidine. 118·1 Mg. of cytidine were dissolved in 15 ml. of 0·5M-sodium hydrogen carbonate, and 7·0 ml. of this solution were treated with 500 mg. of di-(2-chloroethyl)methylamine for 4 days at room temperature, and a further 7·0 ml. of the solution served as a control. Both solutions were then made up to 10·0 ml. with acetic acid, and 2·0-ml. samples of the solution used for estimation of primary amino-groups. Total nitrogen was determined on an acidified solution. Results are given in Table 4.

TABLE 2. *Action of di-(2-chloroethyl)methylamine (X) in* 0·1M-$NaHCO_3$ *on calf-thymus deoxypentosenucleic acid.*

	N, % (± 0·1)	P, % (± 0·2)	Purine-N, % (± 0·15)	Non-purine-N, % (± 0·1)	N/P * (± 0·08)	Deoxy P (± 5%)	Purine N * P (± 0·09)	Amino-N * P (± 0·06)
Nucleic acid B	14·8	9·0	8·75	5·7	3·66	6·6	2·13	0·79
Nucleic acid treated with (*X*) : B/1	14·4	8·15	6·55	8·0	3·92	6·8	1·76	0·79
Nucleic acid : G/2	15·6	9·15	9·5	6·1	3·79	—	2·30	0·79
								0·79
								0·83
Nucleic acid treated with (*X*) : G/2/1	14·4	7·6	6·2	7·6	4·21	—	1·81	0·71
								0·66
								0·72
Nucleic acid : S/1	14·6	8·8	9·1	5·4	3·68	—	2·29	0·82
								0·79
								0·82
Nucleic acid treated with (*X*) : S/1/1	14·6	7·8	6·7	7·6	4·15	—	1·9	0·74
								0·72

* Atomic ratios.

TABLE 3. *Experiment I. Treatment of purine nucleosides with di-(2-chloroethyl)methylamine in* 0·1M-$NaHCO_3$.

	Guanosine		Adenosine	
	Total N, % (± 0·1)	Purine-N, % (± 0·15)	Total N, % (± 0·1)	Purine-N, % (± 0·15)
In water	21·2	20·8	25·3	24·4
In $NaHCO_3$	—	20·7	—	24·3
Treated with amine for 4 days at room temp.	—	9·1	—	20·0
Decrease of purine-nitrogen	—	55%	—	18%

TABLE 4. *Experiment II. Decrease of primary amino-nitrogen in the reaction of adenosine and cytidine with di-(2-chloroethyl)methylamine.*

	Without hydrolysis		With hydrolysis	
	Primary amino-N, mg./ml.	Total purine-N, mg./ml.	Primary amino-N, mg./ml.	Total purine-N, mg./ml.
Adenosine (A)	0·27, 0·25	1·25	0·24	1·34
Adenosine + (*X*) (B)	0·12, 0·11	0·80	0·11, 0·10	0·90
Decrease (%)	63	42	60	41

	Primary amino-N, mg./ml.	Total N, mg./ml.
Cytidine	0·26, 0·24	0·78
Cytidine + (*X*)	0·19, 0·19	—
Decrease (%)	24	—

Results. (i) *Dialysate.* It was shown (Part III, *loc. cit.*) that the product of this reaction contains a proportion of material of low molecular weight. This is confirmed by the fact that on dialysis both organic nitrogen and phosphorus pass through the membrane and a deoxypentose sugar and a purine base can be detected in the dialysate. The total amount dialysing is, however, only about 2% of the starting material. The dialysate is comparatively rich in purines, the atomic ratio of purine-N to -P being 9·5.

(ii) *Inorganic phosphorus.* After treatment with di-(2-chloroethyl)methylamine, as described, the nucleic acid solution contained no inorganic phosphate even after 3 weeks at room temperature. After this period the whole of the phosphorus was still precipitable by 0·25% uranyl acetate in 2·5% trichloroacetic acid (Macfadyen, *J. Biol. Chem.*, 1934, **107**, 299).

(iii) *Analysis of non-dialysable product.* Table 2 shows the analytical results obtained with three distinct preparations, made from different samples of the nucleic acid. The last four columns represent total nitrogen, deoxypentose sugar, purine nitrogen, and primary amino-nitrogen expressed as proportions of the amount of phosphorus present. From the N/P ratios it is found that the amount of residues of the amine attached to the nucleic acid is about one per tetranucleotide in the first preparation and rather more (*ca.* 1·4) in the others. This amount is not known with high precision since the addition of one residue per tetranucleotide causes an increase of the N/P ratio of only 7%, whereas the overall accuracy of the determinations is of the order of ±2%.

The main features of these results are as follows: (1) there is no change in the ratio of deoxypentose sugar to phosphorus as determined by the diphenylamine colour reaction; (2) the amount of purine precipitable as silver salt is diminished by the reaction, and the decrease is significantly greater than the decrease of primary amino-nitrogen determined by the van Slyke method.

It is evident from this that di-(2-chloroethyl)methylamine is capable of reacting with the purine groups of the nucleic acid, and the reaction product survives hydrolysis. The fact that the decrease of purine nitrogen observed is appreciably greater than the decrease of free amino-nitrogen (which includes also whatever effect the reaction of the amine has had on the cytidine amino-group) would appear to show that some reaction with the purines has occurred in other ways than with their primary amino-groups.

(iv) *Riboside experiments.* It is seen (Table 3) that treatment with the amine of both guanosine and adenosine causes an appreciable decrease in the amount of purine-nitrogen. Table 4 shows a comparison of the decrease in primary amino-nitrogen with the corresponding decrease of purine-nitrogen for adenosine with the amine. In view of the fact that the primary amino-determinations of the treated nucleic acid were made after hydrolysis in N-sulphuric acid, it seemed desirable to make the determination with the nucleoside under the same conditions.

It can be seen from the figures that the primary amino-nitrogen determined in the treated nucleoside is the same before and after hydrolysis. It is therefore clear that the decrease in the value of the primary amino-nitrogen caused by the reaction of di-(2-chloroethyl)methylamine is not due to a blockage of the free amino-groups which can be removed by hydrolysis. The decrease of primary amino-nitrogen of adenosine is appreciably greater than the loss of purine-nitrogen under the same reaction conditions. The experiment with the pyrmidine nucleoside cytidine also shows that reaction involving the primary amino-groups has taken place.

The determination of the free amino-nitrogen of guanosine is difficult both on account of its low solubility and because it is known (van Slyke, *J. Biol. Chem.*, 1911, **9**, 183) to give results in the van Slyke apparatus which are 25% too high. No comparison was made in this case.

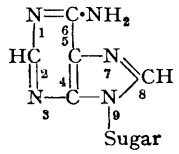
In order to find if the apparent loss of purine was due to a radical change in the purine groups or merely to their substitution, we examined the ultra-violet absorption curves of the solutions of experiment I (above). The measurements were made at a dilution of 33·3 in the medium named. The absorption curves in the region of the main absorption band (230—280 mμ) are not greatly affected, as they might be expected to be if the purine nucleus were changed fundamentally, but the following figures show that maximum of absorption is displaced towards longer wave-lengths by treatment with the amine:

	Maximum of absorption, mμ	pH
Guanosine in $NaHCO_3$ (0·1N)	254	8·71
Guanosine in H_2SO_4 (N)	257	—
Guanosine after treatment with amine in $NaHCO_3$ (0·1N)	270	8·62
Guanosine after treatment with amine in H_2SO_4 (N)	268	
Hydrolysis product of guanosine in H_2SO_4 (N) (guanine)	248	—
Hydrolysis product of treated guanosine in H_2SO_4 (N)	261	—

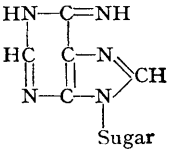
The displacement of the absorption maximum persists after hydrolysis. These displacements would appear to be due to a loading of the purine nucleus with substituents, similar to the effect of the addition of the ribose sugar to guanine. It can be inferred from this, though perhaps not with certainty, that the purine nucleus remains intact.

Discussion

It has been shown that in the reaction of di-(2-chloroethyl)methylamine with the nucleic acid and with the purine ribonucleosides the amount of "purine" precipitable as the silver salt in acid solution is decreased. The extent of the modification of the purine group required to affect non-precipitation in this process is not clearly known. In our experiments it has been found that methylation of guanine in the 1- or the 7-position does not interfere, but in xanthine derivatives methylation in various positions such as the 3- and 7-, and 1-, 3-, and 7-, either completely or partly prevents the precipitation. Although no general rule can be drawn from this, it may reasonably be concluded that the decrease of purine nitrogen observed is due to alkylation of adenosine or guanosine.



(I)



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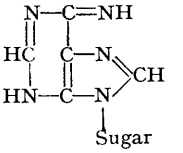

(III)

In the case of adenosine (I), which can also react in the tautomeric forms (II) and (III), it can be seen that every possible reaction involves a loss of primary amino-nitrogen, but only when reaction has occurred in forms (II) and (III) will the ring nitrogen atoms 1 and 3 be alkylated. It is therefore possible in the reaction with adenosine for the decrease of primary amino-nitrogen to be greater than the loss of "purine-nitrogen," as is found to be the case.

On the other hand, with guanosine, there are other possibilities in that (in the keto-form) either of the nitrogen atoms in positions 1 and 3 can be alkylated without the primary amino-group in position 2 being affected. In this case a loss of purine-nitrogen might be possible without any decrease of free amino-group. It would appear from this that the excess of the decrease of purine-nitrogen over that of primary amino-nitrogen in the treated nucleic acid must be due to alkylation of the ring nitrogen atoms of guanosine in this way. It is interesting to note that Fruton, Stein, and Bergmann (*J. Org. Chem.*, 1946, **11**, 559) found that when histidine is treated with various nitrogen "mustards," the attack is on the glyoxaline ring to a greater extent than on the primary amino-group. In the case of cytidine, every possible reaction with di-(2-chloroethyl)-methylamine results in a loss of primary amino-nitrogen, and the reaction occurs to an appreciable extent with free cytidine (Table 4). Since the loss of primary amino-groups in the treated nucleic acid is small (*ca.* 10% of the total present), it follows that the amine can have reacted with the adenine and cytosine groups of nucleic acid to only a very limited extent.

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