Biochemical Pharmacology, 1960, Vol. 4, pp. 34–48. Pergamon Press Ltd., Printed in Great Britain.

THE BIOLOGICAL SIGNIFICANCE OF THE CHANGES PRODUCED IN THE DEOXYRIBONUCLEIC ACID OF CELLS TREATED WITH RADIOMIMETIC ALKYLATING AGENTS*

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Resumé—Les agents alkylants radiomimétiques polyfonctionnels provoquent la formation de pontages (*cross-linkings*) dans le noyau des cellules. Avec les moutardes et une éthylèneimine, une quantité significative de pontages se produit à des concentrations du même ordre de grandeur que celles nécessaires pour inhiber la mitose; ceci n'est cependant pas vrai pour les époxides et les composés du type Myleran qui réalisent des pontages d'une manière très peu efficace.

En ce qui concerne la production de mutations, le pontage ne peut être la réaction essentielle puisque les composés monofonctionnels sont hautement mutagènes. Ces agents alkylants réagissent à la fois avec les groupes phosphates et avec les purines de l'ADN. La comparaison de plusieurs substances permet de conclure que c'est l'estérification des groupes phosphates qui est vraisemblablement la réaction significative et son rôle mutagène est discuté. Après traitement par un époxide monofonctionnel ou par de la moutarde à l'azote, la molécule d'ADN devient plus flexible, de sorte qu'en solution elle occupe un plus petit volume.

Une hypothèse est proposée, selon laquelle les réactions avec l'ADN—pour autant qu'elles aient lieu—ne sont pas responsables de toutes les lésions cellulaires; les combinaisons des agents radiomimétiques avec les groupements acides des composants phospholipidiques des barrières submicroscopiques dans la cellule modifieraient le cytosquelette et interféreraient avec le fonctionnement cellulaire.

INTRODUCTION

THE experiments described in this paper were carried out in an attempt to determine the nature of the initial chemical reactions which are responsible for the biological changes produced by the radiomimetic alkylating agents (see Table 1).

One of the most important clues in the search for the initial receptor (or primary biochemical lesion) is that the essential feature of this class of compounds is the ability to alkylate under physiological conditions. There can be no question that a metabolite of the original drug is involved. Since the chemical constitution of the different alkylating agents varies enormously, the only chemical property common to nitrogen mustards, epoxides ethyleneimines and Myleran-type compounds is that they all combine with electron rich groups as shown in Table 1.† Within

* This work has been supported by grant to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the British Empire Cancer Campaign, the Jane Coffin Childs Memorial Fund for Medical Research, the Anna Fuller Fund and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

† Thiotepa, S:P · $(N\langle^{CH_2}_{CH_2}\rangle)_3$, is a possible exception since its reactivity as an alkylating agent is

TABLE 1. REACTIONS OF NUCLEOPHILIC ALKYLATING AGENTS

Type of compound	Reaction with primary phosphate $\geq P—O—Na^+$	Reaction with amino group $R—NH_2$
Epoxide $CH_2—CH—R$ (O)	$\geq P—O—CH_2—CH(OH)—R$ + NaOH	$R—NH—CH_2—CH(OH)—R$
Ethyleneimine $CH_2—CH_2$ (N, R)	$\geq P—O—CH_2—CH(R)—NH$ + NaOH	$R—NH—CH_2—CH_2—NH(R)$
Nitrogen mustard $Cl.CH_2CH_2N<$	$\geq P—O—CH_2—CH_2—N<$ + NaCl	$R—NH—CH_2—CH_2—N<$ + HCl
Methane sulphonic acid esters $CH_3.SO_2.O.R^1$ (where R^1 = alkyl group)	$\geq P—O—R^1$ + sodium methane sulphonate	$R^1.NHR$ + methane sulphonic acid

TABLE 2. CHANGES PRODUCED IN SERUM ALBUMIN BY RADIOMIMETIC AGENTS*

Treatment	Per cent reduction			
	Carboxyl groups	Amino groups	Imidazole groups	Tyrosine
$O—[CH_2.CH(O)—CH_2]_2$	27	46	77	No reaction
$CH_3.CH(O)—CH_2$	22	60	70	No reaction
Myleran	31	0	42	No reaction
Dimethyl Myleran	16	5	12	No reaction
$COOH(CH_2)_3C_6H_4N(CH_2CH_2Cl)_2$	25	7	5	No reaction

* Three per cent protein solution +0.12 moles of reagent.

any one class of alkylating agents it is possible to have a great diversity of groups attached to the alkylating centre without changing qualitatively the biological effects produced. Nitrogen mustards, which carry an overall negative charge, an overall positive charge, or no charge at all have been prepared and found to be biologically extremely low. Its high biological activity may require the metabolic conversion to TEPA, $O:P \cdot (N(CH_2)_3)$, which still acts by virtue of being an alkylating agent.

active. This excludes the possibility that a specific adsorption process and not an alkylation via the mustard group, is the essential reaction although quantitative differences in activity may be determined by such factors.

The alkylating reaction is not specific, these agents react in the cell with a large number of quite different substrates. In addition, they can and do attack different groups (cf. Table 2) within the same molecule (e.g. protein or nucleic acid). The problem of determining the biologically-relevant site is therefore one of elimination and is quite different from the one normally encountered when studying the mode of action of drugs. Usually drugs interact with very few components of the cell and once these receptors are known the mechanism becomes apparent. We can learn little about the action of the alkylating agents by determining with which molecule they react within the cell. Studies in which the metabolic fate of the alkylating agents is followed in an intact animal are unlikely to provide information about the biochemical lesion since a large part of the administered substance is bound to be wasted in trivial reactions.

It may be possible (ROSS, 1958) to exclude some reactions, notably those involved in sulphydryl groups and amino groups on the basis of a comparison with compounds which readily react with both these groups and which yet do not have radiomimetic properties.*

This points to esterification of anionic groups (carboxyl groups in proteins and phosphate groups in the nucleic acids) as the important reaction. The radiomimetic alkylating agents readily combine with such groups while alkylating agents that are without biological (i.e. radiomimetic) activity combine with amino and sulphydryl groups but do not with anions. Even if this argument is accepted and it cannot be claimed to be conclusive, a great number of different reactions remain and of these probably only a very few are responsible for initiating the biological changes.

A most important fact is the increased activity of polyfunctional (i.e. compounds carrying within the same molecule more than one alkylating centre) over monofunctional compounds. In 1948, HADDOW *et al.* (1948) showed that the tumour growth-inhibiting activity of the nitrogen mustards was confined to polyfunctional compounds only and bifunctional compounds were fifty times more active for the production of chromosome breaks (LOVELESS, 1951). This led GOLDACRE *et al.* (1949), to put forward the cross-linking hypothesis that the alkylating agents join different chromosome threads together by a covalent bond (i.e. cross-linked) prior to mitosis. Our work provides experimental proof for the occurrence of cross-linking though it does not suggest that chromosome threads are so joined. It must be stressed that the cross-linking theory can only be extended to the biological changes which involve interference with cell division as there is increasing experimental evidence (WESTERGAARD, 1957) that many types of mutation are brought about as, or even more effectively by monofunctional than by bifunctional compounds. The cross-linking hypothesis can therefore not be applied to the production of mutations except perhaps those involving chromosome rearrangements.

* War-time research in Belgium, England and the U.S.A. (see review by PETERS, 1947) while showing that mustard gas could inactivate SH enzymes suggested that this was not responsible for its action on the skin. A similar conclusion was reached from cytological data by LOVELESS (1951).

CROSS-LINKING OF DEOXYRIBONUCLEIC ACID (DNA)

Since the most characteristic features of cellular damage by radiomimetic compounds involves nuclear structures it is tempting to postulate that the important initial reaction involves the DNA of the nucleus, and this view was supported by the relatively early work of GJESSING and CHANUTIN (1946) who showed that nitrogen mustard and DNA readily reacted *in vitro*. More detailed studies showed that the alkylating agents which are not radiomimetic, but which do react with sulphydryl and amino groups in proteins do not combine with DNA *in vitro* while all the radiomimetic alkylating agents do (ALEXANDER *et al.*, 1957; STACEY *et al.*, 1958).

In none of the reported investigations involving whole animals (e.g. TRAMS *et al.*, 1959) and using labelled radiomimetic compounds has any evidence been been found for combination with the DNA in the cell and the bound material has usually been found to be linked to proteins. It can be argued that the amount of reaction needed to alter the biological properties of a large molecule like DNA is so small that it has escaped detection.

We have been able to show unambiguously with cells *in vitro* that the alkylating agents react with the DNA in the nucleus. The first system used was with sperm from herring salmon and trout (ALEXANDER *et al.*, 1957, 1959; STACEY *et al.*, 1958).

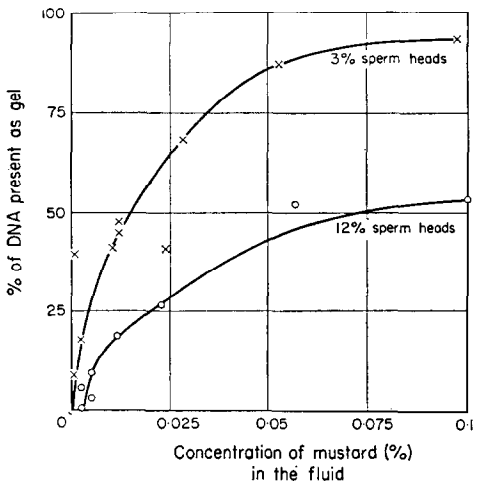


FIG. 1. Cross-linking of DNA within the nucleoprotein of the sperm heads from herring by the phenylalanine mustard (CB 3025). A suspension containing 3 per cent by weight of sperm heads (—X—) and 12 per cent (—O—) was treated with different concentrations of the mustard for 6 hr at 37 °C.

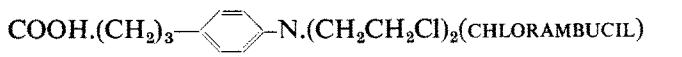
The heads of such sperm are made up essentially of DNA and protamine and disperse completely in 2 M sodium chloride which breaks the salt links between the phosphate groups of the DNA and the basic groups provided by the lysine and arginine side chains of the protamine. After treatment with poly-functional alkylating agents the DNA no longer dissolves completely in 2 M salt.

On visual inspection the sperm heads still appear to disperse, but on centrifuging part of the DNA was spun out as a gel (see Fig. 1). We interpret the production of gel-DNA as the joining together of different molecules via covalent chemical bonds into a very large network. Such a network will swell but cannot go into true solution; as the number of bonds (i.e. cross-links) between the molecules is increased the gel becomes more rigid and swells less.*

Gel first appears when a small fraction of the existing molecules has been linked into what is called "an infinite network". This gel point is reached when, on average, one intermolecular cross-link has taken place for every macromolecule present. Since the distribution of cross-links occurs at random at the gel point, some molecules are not involved in any cross-linking while a few molecules are involved in several cross-links and form the infinite network. Once the number of cross-links exceeds that necessary to attain the gel point the fraction of material in the form of gel rapidly increases. The gel produced in this way contains only DNA and all the protamine remains in solution. This shows that no detectable joining together of the protein and nucleic acid molecules occurs but the possibility that some of the alkylating agents combine with the proteins of the nucleus is not excluded.

The cross-linking reaction is not confined to DNA in sperm heads and we have observed it in a number of different cells (see Table 3). In every case the gel produced consisted only of DNA.

TABLE 3. CROSS-LINKING OF DNA IN CELLS TREATED WITH THE NITROGEN MUSTARD



System treated	Conc. of mustard (%)	DNA present as cross-linked gel (%)
Viable troutsperm in their seminal fluid	0·016	15
E. coli in stationary broth culture	0·05	33
Ascites cells (8×10^8 cells/ml)	0·03	41
Cells from two rat spleens suspended in 10 ml	0·1	39

The test employed in these experiments (namely, measurement of gel fraction) only determines the fraction of DNA which has been cross-linked into an infinite network. It is probably not necessary to cross-link a DNA molecule extensively before rendering it biologically useless, and one cross-link joining two different DNA molecules together may be sufficient to prevent both from taking part in their normal physiological processes. This means that long before the gel point is reached a substantial fraction of DNA molecules are seriously altered. For example, one-sixth of the dose needed to reach the gel point is sufficient to alter by cross-

* The polyfunctional alkylating agents will link together groups in different molecules as well as groups within the same molecule (intramolecular cross-linking). This last process is much the more frequent (ALEXANDER, *et al.*, 1959b) but only the intermolecular cross-linking leads to gel formation.

TABLE 4. COMPARISON OF DIFFERENT MUSTARDS IN CROSS-LINKING 3 PER CENT SUSPENSION OF SPERM HEADS

Name or code no.	Formula	Conc (%)	DNA present as gel (%)
Bifunctional nitrogen mustards			
CB 1385	$(ClCH_2CH_2)_2N$–C$_6$H$_4$–$(CH_2)_2$.CH(COOH)NH_2	0·026	84
		0·008	61
HN2	$CH_3.N.(CH_2CH_2Cl)_2$	0·0098	27
		0·05	74
CB 1045	$(ClCH_2CH_2)_2N$–C$_6$H$_4$–$O.CH_3$	0·094	94
CB 3039	$(ClCH_2CH_2)_2N$–C$_6$H$_4$–$(CH_2)_2N(CH_3)_2$	0·033	63
CB 1644	$(ClCH_2CH_2)_2N$–C$_6$H$_4$–$(CH_2)_2$.CH($COOC_2H_5$)NH_2	0·032	48
Monofunctional HN1	$(CH_3)_2N.CH_2CH_2Cl$	0·11	No detectable gel
CB 1610	$Cl.CH_2CH_2.N(C_2H_5)$–C$_6$H$_4$–$(CH_2)_3COOH$	0·054	No detectable gel
CB 3135	$Cl.CH_2CH_2N(C_2H_5)$–C$_6$H$_4$–CH_2.CH(COOH)NH_2	0·121	No detectable gel

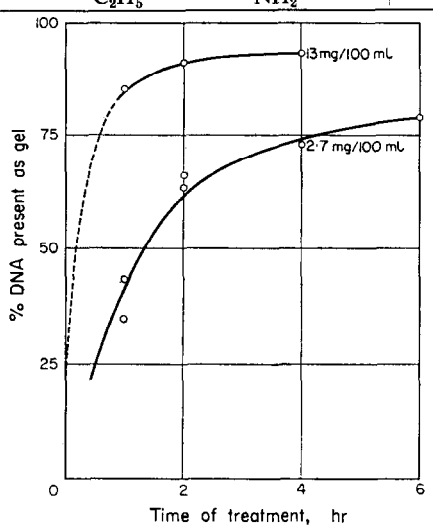


FIG 2. Treatment at 37 °C of a 3 % suspension of sperm heads with the bifunctional ethyleneimine

$CH_2(CH_2)N.CO.NH\ (CH_2)_6\ NH.CO.N(CH_2)CH_2$

(extent of reaction of alkylating agent could not be estimated.)

linking 10 per cent of all the DNA. If this fraction is taken into consideration it can be seen that the amounts of the various nitrogen mustards (see Table 4) and of an ethyleneimine (see Fig. 2) which are needed to "gel" DNA are of the same order of magnitude as those needed to prevent cell division (i.e. inhibition of tumour growth).

The cross-linking hypothesis cannot readily be extended to the Myleran type compound (see Table 5) and the bis-epoxide since these are very ineffective cross-linking agents and the dose needed to produce a measurable amount of gel is many hundreds of times greater than that needed in biological experiments. Although there are differences in the pharmacological action between the different

TABLE 5. CROSS-LINKING OF 3 PER CENT SPERM HEAD SUSPENSION BY ESTERS OF METHANE SULPHONIC ACID

Treating solution	% of gel after different times of reaction 1 hr	6 hr	24 hr
1·6% myleran	2	7	21
0·25% dimethyl myleran*	29	—	—
2·5% myleran	8	19	44
Monofunctional $C_2H_5O.SO_2.CH_3$	—	—	3

* Reacted for 2 hr when more than 90 per cent had reacted either with the water or the nucleoprotein.

classes of alkylating agents on the cellular level, the most striking feature is their similarity and this makes it unlikely that the essential primary chemical processes are greatly different.

NATURE OF THE REACTION OF DNA

If cross-linking of DNA is accepted as a possible mechanism for interference with mitosis the nature of the bonds involved in this cross-linking would only be of secondary importance. For the production of mutations such a reaction is neither necessary nor sufficient and we must therefore look more closely into the groups in DNA with which the alkylating agents react.

On general grounds esterification of the phosphate groups and alkylation of the amino groups and the purines is the most likely reaction. By a titration technique (ALEXANDER *et al.*, 1957) it is possible to distinguish between these reactions since esterification leads to the formation of alkali when epoxides and ethyleneimines are used, but produces neither alkali nor acid when mustards or Myleran-type compounds are used (see Table 1). For reaction with amino groups, the exact reverse is the case. Here acid will be released in the case of the mustards and Myleran-type compounds, while no change in pH will occur on reaction with epoxides or ethylimines. In *in vitro* experiments none of the mustard that had combined with

* The non-reactivity of the amino groups of the purines in DNA is probably due to the fact that they take part in the interchain bonding and are not accessible to the reagents, since other amino reactors such as acylating agents and formaldehyde also do not react with this group in native DNA.

DNA gave rise to acid and we concluded therefore, that all of it was used up by esterification of the phosphate groups (ALEXANDER *et al.*, 1957)*

This deduction, however, was wrong in the case of some of the alkylating agents, as it could not explain the observations made by GJESSING and CHANUTIN (1946) that nitrogen mustard changes the u.v. absorption of DNA. The reaction responsible for this change was identified simultaneously by REINER and ZAMENHOF (1957) and LAWLEY and WALLICK (1957a and b), who isolated from DNA which had been extensively treated with nitrogen mustards or with methyl sulphate, substituted purines which in the case of methyl sulphate were identified as 7-methylguanine and 7-methyladenine. The guanine was much more reactive than the adenine. This reaction, though surprising on general organic grounds since the tertiary ring nitrogen in the imidazole ring is very unreactive, is not in contradiction with the titration data if the 7-methyl derivative, as has been proposed independently by both groups of workers, is only produced by hydrolysis from a quaternary derivative. Quaternization of the tertiary nitrogen, would behave in the titration experiments like an esterification reaction since it does not give rise to the release of acid in the case of mustards.

We have carried out similar experiments (ALEXANDER and STACEY, 1959) using propylene oxide and observe the appearance of a new u.v. absorption maximum at 270 mμ, with an extinction proportional to the amount of reaction (see Fig. 3). In our experiments material absorbing at a maximum near 270 mμ

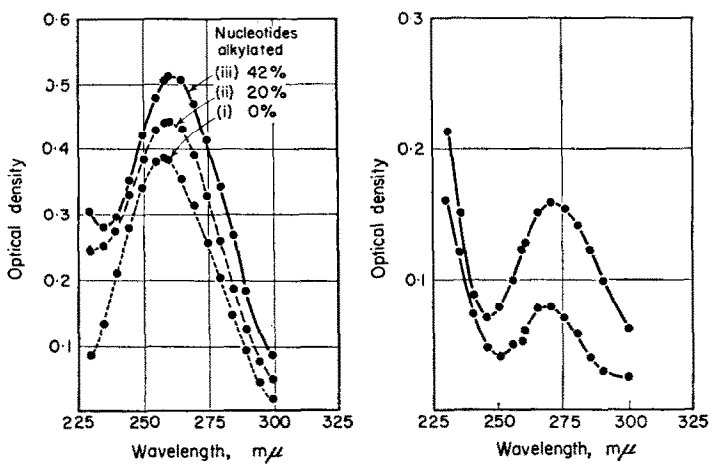


FIG. 3. Change in ultraviolet absorption curve when a 0·1 per cent solution of DNA reacted with propylene oxide (amount of reaction determined from alkali released). Left curve: actual absorption spectrum. Right curve: difference in curves showing new product produced after 20 per cent and 42 per cent alkylation.

can be separated from the DNA by dialysis immediately after the reaction is complete and this suggests that the quaternary compound which is formed as an intermediary is very unstable. The relative amount of phosphate esterification and of purine alkylation varies for different alkylating agents. REINER and ZAMENHOF

(1957) found that alkylation in the 7-position occurred to a very much smaller extent with ethyl than with methyl sulphate and that a nitrogen mustard was intermediate between the two. We have confirmed this observation (ALEXANDER and STACEY, 1959) using different methane sulphonates. Treatment with methyl methane sulphonate is very effective in producing substituted purines while no evidence for their formation can be obtained after treatment with the ethyl compound which, nevertheless, combines with the DNA, presumably entirely with the phosphate groups. Myleran behaves in this respect exactly like its monofunctional equivalent, ethyl methane sulphonate. Esterification is the principal reaction for the very active aromatic nitrogen mustard derived from l-phenyl alanine (CB 3025); so long as the total extent of reaction with the DNA is small—the physiologically-relevant situation—90 per cent or more of the reacted mustard is seen as the ester (ALEXANDER and STACEY, 1959).

Since there seems to be no reason for postulating a different mechanism for the radiomimetic (mutagenic) action of these different compounds the alkylation of the purines would not appear to be involved. This is particularly apparent for ethyl methane sulphonate which is perhaps the most potent mutagenic agent known (cf. various authors at the ERWIN BAUR MEMORIAL LECTURES at GATERSLEBEN, in press), but produces no reaction with the purines in DNA. It is surprising that a compound like methyl methane sulphonate which produces such far-reaching changes in the base composition of DNA should show no unusual biological effects and that it should only differ from the ethyl compound in being a less efficient mutagen.

Pathways by which esterification of DNA can lead to mutations have been discussed by us (ALEXANDER and STACEY, 1959). Possibly to produce a mutant no more than a minor alteration must occur in the "target" DNA so that its biological replication is not prevented, but only slightly interfered with thereby giving an increase in the number of imperfect replicas (i.e. mistakes) that are made. It is this altered DNA made during the course of biological synthesis that represents the mutant. According to this view the alkylation of the DNA does not produce a mutation, but merely increases the chance that a mutation will be produced in the subsequent synthetic processes. If the mutagenic agent modifies the DNA too severely (e.g. cross-links it) it will be "dead" material and new "mutated" DNA will not be produced.

CHANGES IN SIZE AND SHAPE OF DNA

The bifunctional reagents change the physicochemical characteristics of DNA very extensively as a result of cross-linking. At first sight one would not expect reaction of monofunctional reagents to alter the macromolecular properties of DNA, but detailed experiments using the techniques of light-scattering and viscosity show that far-reaching changes do occur, at least, with the two substances so far studied in detail by us, namely propylene oxide and the monofunctional nitrogen mustard derived from l-phenylalanine, $COOH.CH(NH_2).CH_2.C_6H_4.N(CH_2C_6H_5)C_2H_4Cl$ (CB3153).

Light-scattering measures two properties; the molecular weight and the volume which the molecule takes up in solution (expressed as the radius of gyration).

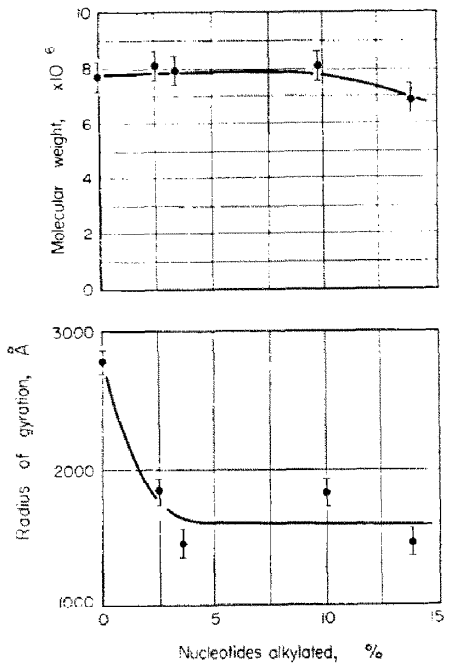


FIG. 4. Changes in the size (molecular weight) and shape (radius of gyration) of DNA molecules following different treatments with the monofunctional mustard CB3153.

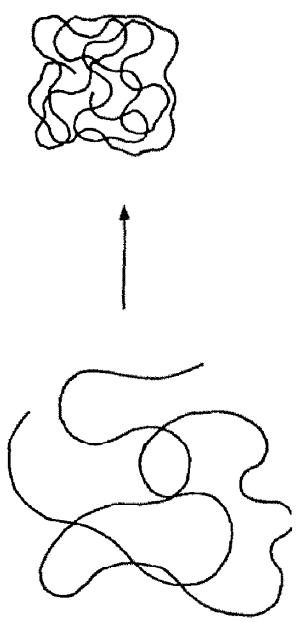


FIG 5. "Coiling" of stiff macromolecule. The length remains the same but because of greater flexibility it occupies a smaller volume.

From Fig. 4 can be seen that the radius of gyration of DNA treated with CB3153 falls without there being any change in the molecular weight.* This means that the molecule is more flexible and can therefore occupy a smaller volume in solution. This is shown diagrammatically in Fig. 5. After extensive reaction which involves more than 10 per cent of the total nucleotide residues the molecular weight falls. Breakdown by hydrolysis of the main polynucleotide chain could occur both as a result of esterification (unstable triesters see (ALEXANDER and STACEY 1959)) or from the elimination of an alkylated purine which leaves a phosphate sugar residue known to be more acid labile.

By whatever mechanism this breakdown occurs it is a rare event—at least for monofunctional reagents—seen only with treatments which are much more extensive than could occur under biological conditions.† Coiling follows either the alkylation or the elimination of the purines which was revealed by the dialysis experiment and which will make the stiff double helix more flexible. Esterification of phosphate groups could produce coiling by reducing the electrostatic charge on the molecule which contributes to its stiffness, but changes in shape by this mechanism would only set in when more than 20 per cent of the phosphate groups have been esterified (KUHN *et al.*, 1948) while with propylene oxide and the one-armed mustard coiling is already observed after a few per cent of reaction.

IS DNA THE SITE OF THE PRIMARY REACTION?

The experiments described above have shown that the radiomimetic alkylating agents produced far-reaching changes in the chemical and physical properties of DNA. The mere fact that such changes occur does not, however, prove that they are the significant reaction which gives rise to the observed biological end-effects. The attraction of relating mitotic inhibition to cross-linking of DNA, and mutagenesis to esterification of the phosphate group of DNA (see p. 40) is that the point of the initial chemical reaction coincides with that of the biological end-effects (i.e. most of the lesions involve the nucleus because it is the DNA which is alkylated). Yet in the case of ionizing radiations for which a similar argument could be advanced no such conclusion can be drawn. Like the alkylating agents, ionizing radiations modify chemically many cell components including the cross-linking of DNA in nucleoproteins, but these reactions with DNA do not, for many of the radiation effects, fulfil the requirements which the primary chemical lesion has to show (ALEXANDER *et al.* 1959a).

* There is a delay between the end of the alkylating treatment and the actual measurement, and it is possible that some of the changes observed take place as an after-effect during the period needed for reaction and analysis. In the experiment shown in Fig. 4 this amounted to 8 hr at 4 °C. The sample, however, does not alter on being allowed to stand for a further 24 hr at room temperature.

† Measurements of the viscosity of DNA solutions have often been used to follow changes. While viscosity is very useful as a quick and simple method for revealing that there has been an alteration, it cannot distinguish between coiling and main-chain breakage. Viscosity measures the volume which a molecule occupies in solution. This volume may be reduced by main-chain scission or by rendering the molecule more flexible so that it can coil up more tightly. Failure to appreciate that a drop in viscosity can be due to these two quite different reactions has led to the claim (BUTLER, 1950) that the principal reaction of nitrogen mustards is to degrade DNA. The first change produced by nitrogen mustards is a coiling up. Degradation only occurs after more extensive reactions and after the viscosity has fallen to a very low value due to the earlier coiling reaction.

BACQ and ALEXANDER (1955) were led to speculate some years ago on the possibility that a primary biochemical lesion of irradiation involved the breakdown of the submicroscopic intracellular barriers—the cytoskeleton. This may result in the release of enzymes from sites from which they are normally confined (such as by adsorption on to surface films at interphases) and enable them to attack substrates from which they are normally separated. In the intervening years this enzyme-release theory has been strengthened by many biological investigations and it has become apparent that damage to nuclear structures need not be initiated by ionizations that have occurred within the nucleoproteins.

In the case of the alkylating agents a similar mechanism may well be operative and this possibility was envisaged 12 years ago by PETERS (1947) in a review on the action of mustard gas. More recently, PETERS (1956) suggested that the function of hormones was to modify or reorientate the cytoskeleton, which is the co-ordinating factor in the activity of the cell. A characteristic of hormones is that they act on the cell as a whole and do not influence any one enzymatic step in particular.

The nature of the internal membranes in cells was first discussed by PETERS in 1929 and his predictions have been fully confirmed by the recent electron-microscopic investigations. PETERS postulated, and no better theory has been advanced in the intervening years, that the internal membranes are made up in part of surface-active materials which carry an electric charge. Enzymes are absorbed on to them. The principal charges in the phospholipids are anionic groups with which the alkylating agents react readily under physiological conditions. That alkylation of the acid groups on membranes will take place when the radiomimetic agents are introduced into cells is a most reasonable prediction which we propose to test in model systems. The failure of substances which can alkylate amino groups and sulphydryl groups under physiological conditions, but which cannot esterify acid groups, to have radiomimetic properties, could be explained equally well if the important sites for reaction are the acid groups of intracellular membranes or the acid groups of the nucleic acids.

SUMMARY

Experiments are described which show that the polyfunctional radiomimetic alkylating agents cross-link the DNA in the nucleus of cells. With the mustards and an ethyleneimine a significant amount of cross-linking occurs at concentrations of the same magnitudes as those needed to inhibit mitosis but this is not the case for the epoxides and for Myleran-type compounds which cross-link very ineffectively.

For the production of mutations cross-linking cannot be the essential reaction as monofunctional compounds are highly active. Reaction of these alkylating agents occurs both with the phosphate groups and with the purines of DNA. From a comparison of different substances it is concluded that esterification of the phosphate groups is more likely to be the significant reaction and its role in mutagenesis is discussed. After treatment with a monofunctional epoxide and nitrogen mustard the DNA molecule is made more flexible so that it occupies a smaller volume in solution.

The possibility is envisaged that reactions with DNA—though they occur—are not responsible for some or all of the cellular lesions. Combinations of the radiomimetic agents with the acid groups of the phospholipid components of the submicroscopic barrier in the cell could modify the cytoskeleton and interfere with the functioning of the cell.

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DISCUSSION

R. THOMAS: Vous avez fait allusion à un accroissement de la flexibilité des molécules d'ADN après la perte de bases puriques consécutives à la méthylation. Pensez-vous que ceci puisse être attribué à ce que la libération de chaque base purique s'accompagne de la labilisation d'une liaison phosphoester contiguë?

P. ALEXANDER: I think it is very likely that ester hydrolysis contributes to the increase in flexibility but limited ester hydrolysis without elimination of the base does not greatly change the flexibility.

C. LIÉBECQ: I should like to know whether anyone has already tried to see what happens in the presence of KORNBERG'S DNA synthetizing enzyme if DNA esterified by ethyl-methane sulphonate is added as a primer instead of normal DNA.

P. ALEXANDER: As far as I know this experiment has not been done.

F. KASTEN: You indicated that treatment of DNA with alkylating agents causes an alkylation of the purine bases, a loss of purines, and a consequent coiling up of DNA. Would you expect that following mild acid hydrolysis, as in the Feulgen reaction, nuclear chromatin would show a different distribution pattern compared with chromatin whose purines are not lost?

P. ALEXANDER: Yes; the loss of purines will cause coiling. But the acid treatment will also cause hydrolysis of phosphate ester groups and the macromolecular properties will therefore be entirely altered.

J. N. DAVIDSON: (a) From your point of view would the action of nitrous acid be too drastic. (b) If the effect of nitrous acid were sufficiently mild to deaminate only a few bases in the DNA chain, would it meet your requirements of a mutagenic agent?

P. ALEXANDER: The mutagenic action of nitrous acid—so long as the reaction is confined to deamination—fits in well with the mechanism proposed for the alkylating agents since it produces a minor modification in the DNA only and probably does not interfere with its physiological function. Like esterification it merely provides a slightly imperfect template thereby increasing the chance of copy-error during synthesis. Complete elimination of a base would appear to be too drastic and probably prevents subsequent duplication at least of the part of the macromolecule which has undergone reaction. It is the elimination of the base following alkylation which prevents methyl methane sulphonate from being a good mutagen. On the other hand, deamination by nitrous acid leaves the modified purine in the molecule.

T. CASPERSSON: Have you made any observations on changes in what you called "flexibility" of DNA in solution irradiated with small doses of (a) ultraviolet: (b) ionizing radiation.

P. ALEXANDER: The first change which is seen when DNA is irradiated in solution with u.v. of 254 mμ (in the absence of oxygen) is coiling (i.e. the molecular weight remains unchanged while the viscosity and the radius of gyration falls). As the treatment with u.v. is increased then the molecular weight also falls.

With ionizing radiations we have never seen coiling. In dilute solutions of DNA when the action is indirect (i.e. due to OH radicals) the molecule is "chopped"

but its rigidity unchanged. When DNA is irradiated under conditions when the action of ionizing radiations is direct then the end effect depends on the exact conditions of the DNA. When tightly packed, as in the sperm heads of fish, then cross-linking of the DNA occurs especially in the absence of oxygen. In swollen nuclei where the DNA strands are separated then degradation to lower molecular weight occurs but dissolved oxygen may have to be present. We believe that following ionization the affected molecule can either cross-link or degrade depending on conditions. A similar situation has been encountered by us with some synthetic polymers and several mechanisms can be proposed.

J. BRACHET: How do you visualize the effect of your phosphate esterification reaction on the synthesis of a specific protein? Would it not be easier, in view of present ideas on the mechanism of genetic information transfer, to imagine a minor change in one of the bases, as in the case after HNO_2 treatment?

P. ALEXANDER: I do not believe that the esterification results in a change of function of the DNA. Indeed I postulate that the alkylated DNA is *not* the mutated DNA. I suggest that a minor change which left the physiological function of the DNA uneffected, i.e. it still controls protein synthesis and it is still capable of acting as a template for DNA synthesis. But the slight imperfection that has been introduced increases the chance of "copy-error" during duplication. It is the occasional "copy-error" which occurs in synthesis which gives the mutated DNA that gives rise to a different specific protein.

A. LOVELESS: The survival of starved cells of *E. coli* B. in solutions of ethylene oxide or methyl methane sulphonate has been found to be independent of the dose rate, i.e. the concentration of the compound applied, so long as the product of concentration and time of treatment was kept constant.

By contrast, ethylmethane sulphonate was far more effective when given in high concentration for a short time than when given in lower concentration for a correspondingly longer time. Since all platings were made immediately upon termination of treatment, this argues that the chemical reaction is spontaneously reversible. Whether this is in fact the case will be tested by keeping the cells, after various treatments, for the same overall duration in non-nutrient medium prior to plating.

In any event, the observation argues an entirely different reaction on the part of methylating and hydroxyethylating agents on the one hand and ethylating agents on the other; this difference may be explicable in the manner suggested by Dr. Alexander.

Z. M. BACQ: The situation described by Dr. LOVELESS for two different alkylating agents also exists with ionizing radiation. It is well known that in *Drosophila* the increased rate of mutation induced by X- or γ-rays is not dose-rate dependent. On the contrary in mice, RUSSELL has recently demonstrated that the mutagenic effect of γ-radiation is dose-rate dependent, i.e. one obtains twice to three times more mutations if a given dose of γ-rays is administered in a single exposure instead of being distributed over some weeks.