Biochemical Pharmacology, 1960, Vol. 4, pp. 49–56. Pergamon Press Ltd., Printed in Great Britain.

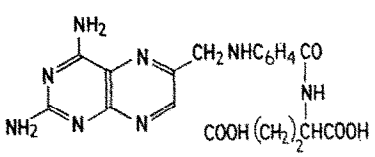
THE ACTION OF ANTIMETABOLITES AND BIOLOGICAL ALKYLATING AGENTS ON THE SYNTHESIS OF DEOXYRIBONUCLEIC ACID AND A POSSIBLE RELATION BETWEEN THE MECHANISMS OF ACTION

G. M. TIMMIS

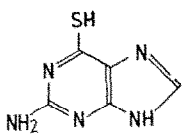
The Chester Beatty Research Institute,
(Institute of Cancer Research, Royal Cancer Hospital) London, England

Résumé—Dans cette communication, le mécanisme d'action des antagonistes de l'acide folique, des purines et des pyrimidines, ainsi que l'action de l'O-diazo-acétyl-L-sérine (azasérine) et de la 6-diazo-5-oxo-L-norleucine, sont discutés par rapport particulièrement à leur capacité d'agir en antimétabolites irréversibles. L'auteur démontre aussi la possibilité que les composés du type "alkylant" agissent en se combinant d'abord avec la guanine de l'acide nucléique; puis la guanine alkylée peut se scinder et agir en antimétabolite irréversible et trés puissant. Les différences marquées entre l'action biologique du Myleran et celle des ypérites sont discutées, et des explications possibles de ces différences sont proposées.

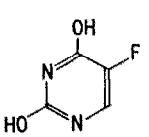
THE antimetabolites which affect nucleic acids synthesis comprise the antifolics, antipurines, antipyrimidines and the two antibiotics, azaserine and 6-diazo-5-oxo-L-norleucine (D.O.N.) The most effective antifolics have a pteridine structure, e.g. aminopterin (I) and amethopterin, and interfere with nucleic acids synthesis by preventing the conversion of folic acid to tetrahydrofolic acid and folinic acid; inhibition of this process prevents the provision of "one carbon" units for the synthesis of purines. The mechanisms of action of the antipurines such as 6-mercaptopurine or 2-amino-6-mercaptopurine (II) are less certain, but probably one important reaction may lie in the direct conversion of the antimetabolite to its ribotide, which then interferes with utilization of normal purine ribotides. More is known about the



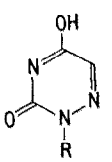
I



II



III

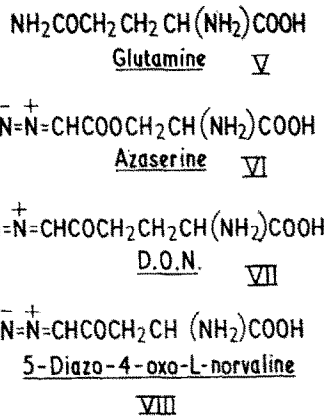


IV

R = Ribose

FIG. 1.

two most generally interesting antipyrimidines, namely 5-fluorouracil (III) and 6-azauracil riboside (IV). They act, respectively, by inhibiting the methylation of deoxyuridylic acid to deoxythymidylic acid (HEIDELBERGER *et al.*, 1958) and by preventing the normal decarboxylation of orotidylic acid to uridylic acid (HANDSCHUMACHER and PASTERNAK, 1958). In all these cases the antimetabolite and the corresponding metabolite show the expected analogy in structure and in most cases investigation has led to the discovery and isolation of the essential enzyme which is inhibited by the antimetabolite. With the possible exception of aminopterin, inhibition is reversible by addition of an excess of the metabolite. Azaserine (O-diazoacetyl-L-serine) (VI) and D.O.N. (6-diazo-5-oxo-L-norleucine) (VII) are again antimetabolites with structures obviously analogous to the corresponding metabolite glutamine (V), which they prevent from aminating formylglycineamide ribotide (IX) to formylglycineamidine (X) (R = ribotyl) (LEVENBERG *et al.*, 1957). In



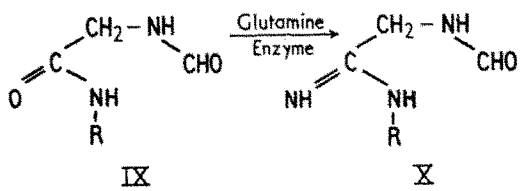


FIG. 2.

addition, they are particularly interesting because, while inhibition of the essential enzyme by the antagonist can to some extent be annulled by the addition of glutamine, there is no doubt that there is a significant amount of irreversible inhibition, that is to say, the enzyme or co-enzyme is permanently deprived of its function by stable combination with the antimetabolite. There is little doubt that the diazoalkane moiety of the antagonists is responsible for this bonding, by alkylation. 5-Diazo-4-oxo-L-norvaline (VIII) although closely related in structure to D.O.N. did not inhibit the enzyme; again, other enzymes were very feebly, if at all, inhibited by azaserine and D.O.N. (BUCHANAN, 1958). We have therefore a clear demonstra-

antimetabolite and enzyme or coenzyme and, in addition, the utility of an alkylating group which renders the antagonist irreversible under the right conditions. These would require that the alkylating group in the antagonist molecule would be favourably positioned in relation to the site to be alkylated in the enzyme or coenzyme so that during the process of attachment the alkylation would be sterically favoured. It is probably this last factor which makes D.O.N. very much more active as a tumour inhibitor than the other mainly reversible antagonists, e.g. the antifolics, antipurines and antipyrimidines. D.O.N. is as active as triethylenemelamine or one of the more active nitrogen mustards. Recently it was shown that alkylating agents can combine with the nucleotides of the two purine bases (guanine and adenine) and one of the pyrimidine bases (cytosine) which occur in nucleic acid. Alkylation of the guanine moiety was considerably more facile than that of cytosine, and adenine showed the least avidity for the alkylating agent. Alkylation of deoxyguanylic acid (XI), leads via a quaternary salt (XII), which decomposes at pH 7, to the formation of the corresponding 7-alkylguanine (XIII, R = alykl). When guanylic acid is alkylated, a rather more stable, exactly analogous, quaternary salt is formed which is, however, readily decomposed at pH 8–9 to yield the pyrimidine derivative (XIV) (LAWLEY, 1957). Very recently DNA (deoxyribonucleic acid) (1% solution in phosphate buffer) has been treated with sulphur mustard ($ClCH_2CH_2SCH_2CH_2Cl$) heavily labelled with ^{35}S at a level of about 3 moles of drug to 1 mole of the nucleic acid i.e. 0·001 mole of mustard per mole of nucleotide on the assumption of a molecular weight of about 10^6 for DNA. After 10 min the mustard was completely bound and the bases were isolated from the nucleic acid. By chromatography and autoradiography it was proved that alkylation had been nearly all confined to the guanine moiety, the product being (XVI). This compound is evidently derived, by hydrolysis during manipulation, from the initially formed (XVII). This 7-alkylguanine, also prepared by the alkylation of guanosine (XV) in glacial acetic acid solution, was obtained as a pure compound which was analysed and characterised. If the alkylated solution of DNA was allowed to stand, the same product could be isolated from the solution and also a relatively smaller quantity of a different substance was precipitated; its relative insolubility, the fact that it contained ^{35}S and would not run on the chromatogram and the similarity of its u.v. spectrum to that of (XVI), suggested a bis-guanyl compound derived from 2 moles of guanine and 1 mole of the mustard.

RNA (ribonucleic acid) isolated from tobacco mosaic virus was similarly alkylated with sulphur mustard and again the major product was the 7-alkylguanine; in this case the reaction solution did not deposit a precipitate, as was to be expected from the previous result of the alkylation of guanylic acid when a relatively stable quaternary salt was formed. The alkylation of tobacco mosaic virus, even at a dose of very approximately 1 mole of the mustard to 1 mole of RNA (calculated in the same manner as for the DNA experiment) again yielded the 7-alkylguanine and finally *in vivo* experiments, using *B. megatherium.* and a mouse ascites tumour clearly demonstrated the alkylation of the guanine moiety in both the DNA and RNA. In all cases where the relative quantities could be estimated the amount of the 7-alkylguanine found was considerably greater than the product derived from 1 mole of the mustard and 2 moles of guanine; similarly the alkylation of guanosine even

with a considerable excess of the mustard yielded more of (XVI) than the presumed bis-guanyl compound. The view is therefore confirmed that *in vivo* the major initial product of alkylation of DNA will be (XVII). This structure contains both a modified purine structure and an alkylating moiety; it could therefore act as an antimetabolite for normally functioning purine derivatives and, in addition, the alkylating function could render the inhibition irreversible. The analogy with

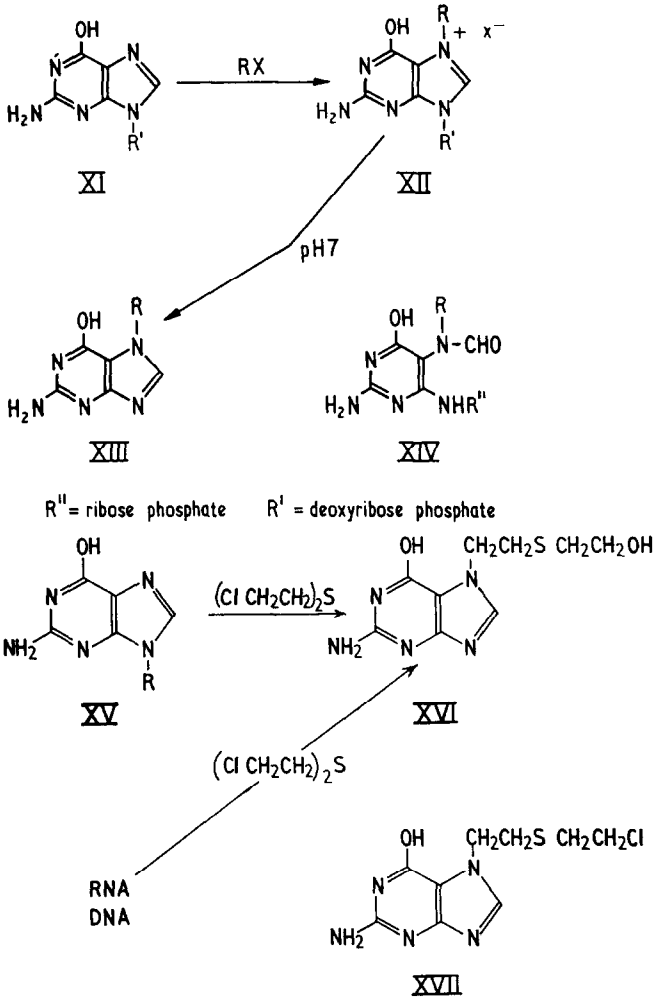


FIG. 3.

azaserine or D.O.N. strengthens the postulation that the bifunctional alkylating agents in general could exert their inhibiting effect on DNA synthesis and growth, at least in part via an antimetabolite action. As an example of the enormous effect that can be exerted by irreversible antagonists in favourable conditions the case of the inhibition of choline-esterase by di*iso*propylfluorophosphonate may be mentioned though this, of course, involves phosphorylation (WEBB, 1948). There

is some circumstantial evidence available, in the case of Myleran, $CH_3SO_2O(CH_2)_4OSO_2CH_3$, for the antimetabolite hypothesis. The effect of Myleran, and also Dimethyl myleran, on the blood elements is remarkably similar to that of the anti-purine, thioguanine (Fig. 4). The action of Myleran on rat bone marrow was investigated in great detail and yielded the striking conclusion that it caused a considerable lengthening of the intermitotic interval of the blood cells in contrast

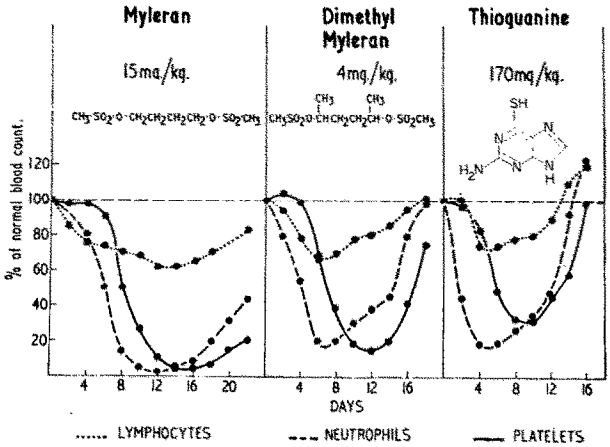


FIG. 4. Blood response of rats to single doses.

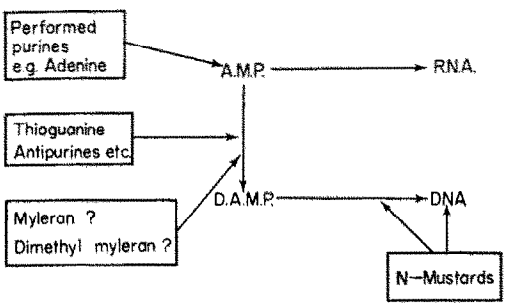


FIG. 5.

to the effect of the nitrogen mustard, Chlorambucil, $(ClCH_2CH_2)_2NC_6H_4(CH_2)_3COOH$, (CB 1348) (ELSON *et al.*, 1958). This effect could also be used to explain the fact that in experiments on the fertility of rats it was found that when they were treated with Myleran its infertilizing effect was delayed for from 6 to 8 weeks in contrast with other alkylating agents where the effect was exerted much earlier (JACKSON *et al.*, 1959). The diagram in Fig. 5 illustrates in a simplified and inevitably speculative way, the nucleic acid synthesis situation in the intermitotic interval, or resting stage (ELSON, 1959). It is thought that an increase in RNA synthesis would increase the intermitotic interval. Those antipurines, such as thioguanine, which interfere in the "preformed purine" pathway of synthesis appear to exert their inhibiting function on the adenosine monophosphate to deoxyadenosine monophosphate pathway and the result of their inhibiting effect on DNA synthesis would be to increase RNA synthesis; it is suggested that Myleran acts similarly in

accordance with its ability to lengthen the time between mitoses. When patients with leukaemia are treated with nitrogen mustards or triethylenemelamine the excretion of uric acid is further increased, but on the other hand treatment with Myleran does not have this effect and it was suggested that the mustards destroy leucocytes, thus accounting for the extra production of uric acid from the purine moieties of the nucleic acid. Conversely Myleran acts more to inhibit mitosis in leukaemic tissue,

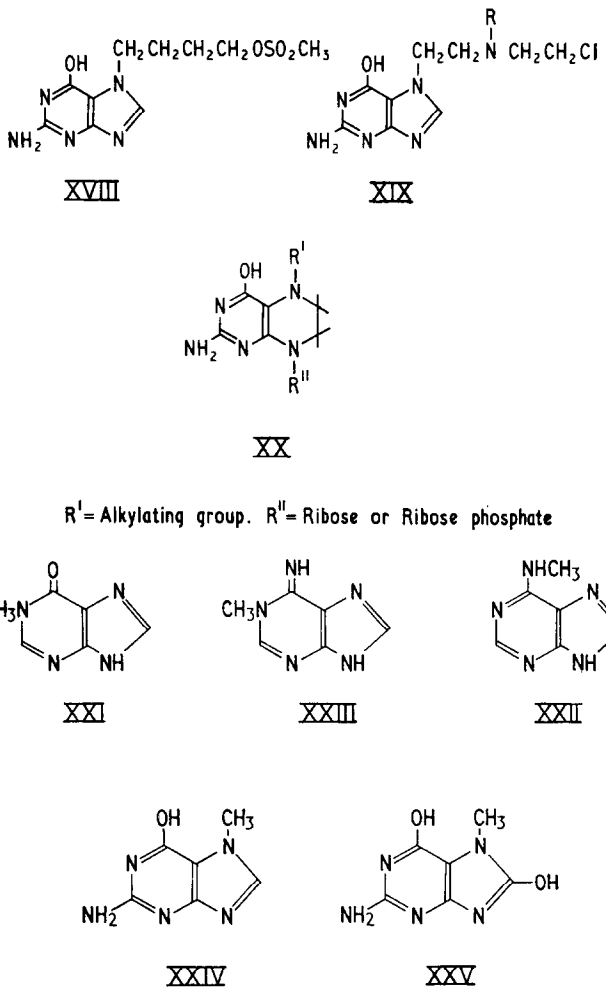


FIG. 6.

by interference with biosynthesis, rather than to destroy it (WINKLER *et al.*, 1957). In considering the antimetabolite hypothesis for the mechanism of action of Myleran in so far as it involves an antipurine type of action it must be remembered that we have as yet no evidence for alkylation *in vivo* of a purine moiety of nucleic acid by the drug. The evidence from *in vitro* studies indicates that alkylation of phosphate groups occurs appreciably (ALEXANDER and STACEY, 1959) and we have preliminary evidence also that alkylation of the guanine moiety in DNA occurs,

but to a much less extent than with the mustards. It is, however, quite possible that guanine alkylation could be catalysed, analogously to the *in vivo* methylation of the ring nitrogen in nicotinamide. This is effected by adenosyl-methione ("activated methionine") (CANTONI, 1953) and a precisely analogous intermediate with a highly active alkylating potential might be formed from Myleran and homocysteine. The methylation of 2:6-diaminopurine to 6-amino-2-methylaminopurine by means of adenosylmethionine has been demonstrated (REMY, 1959). The marked differences between the biological effects of Myleran and the mustards, which has been shown in experiments on the rat peripheral blood and in other respects (ELSON, 1955), (Table 1), could be explained by assuming a destructive attack of the latter on the

TABLE 1.

Haematological effects	CB 1348 12·5 mg/kg body wt.	Myleran 15 mg/kg body wt
Type of action	Rapid, shortlived, destructive, unselective	Slow progressive, inhibitory, dissociated
Lymphoid	Marked depletion	None
Myeloid	Rapid destruction followed by rapid regeneration	Slow inhibition Slow regeneration
Erythroid	Shortlived hypoplasia, transient peripheral effect only	Inhibition lasting 2 days
Platelets, megakaryocytes	Shortlived depletion Brief thrombocytopenia	Prolonged, profound depression with thrombocytopenia
Regeneration	Rapid, immediate, uniform	Slow, patchy, dissociated

blood cells, especially the lymphocytes. In this process damage to DNA most probably plays a large part, but there are also obvious opportunities for formed antimetabolites to affect DNA synthesis at various points. We can now consider how chemical differences in the potential antimetabolites which could be formed from Myleran and the mustards might also contribute to the biological differences. Turning now to the irreversible antagonists which could be derived from this pair of drugs (XVIII and XIX, respectively) there is an important difference between them in the mechanism of their alkylating action which could be reflected in their biological actions. Myleran alkylates mainly by means of a direct displacement reaction and the rate of reaction will depend upon its concentration and that of the group which it alkylates. The nitrogen mustard will react *via* the intermediate formation of a carbonium ion and the rate of reaction will depend very little upon the concentration of the group alkylated. Thus the amount of the potential antagonists formed could be quite different; in addition the reactions of the alkylating moieties of these derived antagonists would be governed by the same factors which apply to the original drugs, and a further cause for disparity could thereby be introduced. There is a possibility that, *in vivo*, alkylation of RNA might yield an alkylated derivative (cf. XIV) of pyrimidine. This compound could be available (cf. WEYGAND and WALDSCHMIDT, 1955) for the synthesis of tetrahydropteridines of the type (XX) possibly still containing an alkylating group, which might be antifolics as such, or after loss of sugar and oxidation to the dihydro form. Adenylic acid on alkylation with dimethyl sulphate gave the

1-methyl derivative which at alkaline pH was converted to the ribotide of (XII) a rearrangement which might nevertheless occur *in vivo*. This methylating process might be responsible for the occurrence of (XXI) (isolated from urine) (WEISSMANN *et al.*, 1957) and (XXII) which has been isolated from RNA fractions (DUNN and SMITH, 1958). They could be derived from (XXIII) by hydrolysis and by rearrangement. Analogously perhaps (XXIV) and (XXV) which occur in urine are the products of methylation *in vivo* of the guanine of nucleic acid or its precursors. The function of these abnormal purines is unknown but may be important since they may occur as units of abnormal nucleic acids and the possibility remains that the biological alkylating agents may interfere with the formation of these bodies.

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DISCUSSION

J. DANIELLI: What do you think happens when nucleic acid is ethylated by ethyl methane sulphonate?

G. M. TIMMIS: There is no doubt that under the conditions of *in vitro* experiments and even using a relatively large excess of the agent there would be very much less alkylation of the guanine moiety than occurs when a mustard e.g. sulphur mustard is used. This follows from our experience with Myleran. As suggested in my paper for the case of Myleran, it seems possible that, *in vivo*, ethyl methane sulphonate might to some extent ethylate homocysteine and the ethionine formed then pass to an "activated" form (compare "activated" methionine which is adenosylmethionine) which would be more likely to alkylate the guanine moiety in nucleic acid than the original agent. However, if this were the case, one would not expect an antimetabolite effect to be shown since the formation of an irreversible antagonist would of course be ruled out.