The Effect of Nitrogen Mustards on the Incorporation of Amino Acids into Protein by *Staphylococcus aureus*

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Nitrogen mustards have been used clinically with some success in the treatment of malignant lymphadenopathies and the chronic leukaemias (see, for instance, Galton, Israels, Nabarro & Till, 1955), and they are also effective in inhibiting the growth of some solid tumours in experimental animals. Their biochemical mode of action remains obscure, although many studies *in vitro* have shown that they readily enter into chemical combination with nucleic acids and proteins under very mild conditions (reviews by Alexander, 1954 and Ross, 1952; also Cohn & Crathorn, unpublished work). More recently, Cohn (unpublished work) has found that *p*-di-(2-chloroethyl)amino-DL-phenylalanine becomes firmly bound to the proteins of liver and other tissues when the compound is injected interperitoneally into rats.

The present investigation represents an attempt to determine the extent to which the nitrogen mustards exert their effects on the direct incorporation of amino acids into proteins. A bacterial system was selected because of its simplicity. The use of *Staphylococcus aureus* offered a further advantage: only in this organism have two modes of amino acid assimilation been demonstrated, one associated with net protein synthesis and the other with an 'exchange' reaction between free amino acids and the amino acid residues within the preformed protein molecules (Gale & Folkes, 1953*a*).

METHODS

Organism. The organism used throughout this work was *Staphylococcus aureus* strain Duncan (Gale & Folkes, 1953*b*), which was kindly supplied by Dr K. McQuillen. It was maintained by frequent subculture on solid peptone–agar slopes. Larger batches for experimental purposes were obtained by growing the organism on a liquid medium of the following composition (%): glucose, 1; Marmite, 0·5; peptone, 1; NH_4Cl, 0·2; Na_2HPO_4, 0·6; KH_2PO_4, 0·3; NaCl, 0·3; $Na_2SO_4,10H_2O$, 0·025; $MgCl_2,6H_2O$, 0·0085 (all w/v). At 37° on this medium the yield of cells was approx. 1 mg. dry wt./ml. when grown for 16 hr. in Roux bottles.

Preparation of suspensions and conditions for study of uptake of ^{14}C*-labelled amino acids.* The bacteria were harvested after overnight growth and washed with water. Suspension densities were determined from the absorption at 420 mμ on a Unicam spectrophotometer (Model SP. 600) previously calibrated against the organism used. Thick suspensions of the organism (50 mg. dry wt./ml.) were then prepared in 0·05M-phosphate buffer (prepared from KH_2PO_4, brought to pH 7 with NaOH) containing 1% (w/v) glucose, and 5 ml. samples were pipetted into each flask; the nitrogen mustards to be studied were added to the individual flasks at this stage. After aerobic incubation, while shaking at 37° for 0·5 hr., the ^{14}C-labelled amino acid was added, and the incubation continued for a further period of 90 min. In this way the labelled amino acid was taken up under exchange conditions. In experiments where an investigation was made of the effects of nitrogen mustards on uptake under conditions of net protein synthesis the bacteria were collected and transferred to an inorganic salts–glucose medium containing a complete mixture of amino acids. The labelled amino acid was then added and the incubation continued as in the experiments carried out under exchange conditions. The incubation was stopped by adding aq. trichloroacetic acid to a final concentration of 5% (w/v) and cooling to 2°. The precipitated proteins were collected and extracted first with water–ethanol (1:3) at 37° for 18 hr. and then with water–ethanol–ether (1:3:4) at 37° for 1 hr. The nucleic acids were then removed by two treatments at 95° with 5% (w/v) trichloroacetic acid. The proteins were finally washed twice with acetone and dried at 70°.

Toxicity tests. The incubations of buffered bacterial suspensions with nitrogen mustards were carried out at 37° for periods similar to those used in the examination of the uptake of amino acids by the cells. Viable cell counts were then made on peptone–agar plates after serial dilutions of the bacterial suspensions with sterile water. These dilutions had reduced the viable counts to the order of a few hundred/plate in control suspensions untreated with nitrogen mustard.

Assays of radioactivity. These were carried out on 'infinitely thick' samples of protein plated on 1 cm.^{2} polythene disks (Popják, 1950), an EHM 2S end-window Geiger–Müller counting tube supplied by G.E.C., London, being used in conjunction with a type 100C counting unit manufactured by Panax Equipment Ltd., Mitcham, Surrey. The counter was housed in a lead castle manufactured by E.R.D. Ltd., Slough, Bucks. Under these conditions a ^{14}C-labelled protein sample of specific activity 1 μc/g. would give an observed counting rate of about 600 counts/min. when plated at 30 mg./cm.^{2} (i.e. a counting efficiency of 1·2%).

Materials. [1-^{14}C]Glycine was supplied by the Radiochemical Centre, Amersham, Bucks. DL-[3-^{14}C]Phenylalanine was synthesized in these laboratories by Dr V. C. E. Burnop.

Details of the syntheses of the nitrogen mustards used are to be found in the recent literature (Everett, Roberts & Ross, 1953; Bergel & Stock, 1954; Davis, Roberts & Ross, 1955; Bergel, Everett, Roberts & Ross, 1955*b*).

Tumour-inhibition tests. (Personal communication from Professor A. Haddow.) Tumour-growth-inhibition tests by nitrogen mustards were carried out on the transplanted Walker rat carcinoma (Badger, Elson, Haddow, Hewett & Robinson, 1942; Haddow, Harris, Kon & Roe, 1948). Most of the results quoted here have been briefly reported elsewhere (Everett *et al.* 1953; Davis *et al.* 1955; Bergel *et al.* 1955*b*).

RESULTS

The initial experiments were carried out with three aliphatic nitrogen mustards: dimethyl-(2-chloroethyl)amine (M1), methyldi-(2-chloroethyl)amine (M2) and tri-(2-chloroethyl)amine (M3) (see Table 1). M2 is widely used in the treatment of leukaemia and allied diseases, it being often preferred to M3, which is rather more active but also more toxic than M2 (Galton, private communication). It was found (Table 1) that the inhibition of the amino acid-exchange reaction in our system paralleled the therapeutic efficiency of the compounds, the inhibition of incorporation being greater than would be explained by the reduction in viability of the organism. In a further experiment, the uptake of amino acids by the M3-poisoned system was studied under conditions where amino acid exchange could be compared with the uptake of amino acids under conditions otherwise favourable for net protein synthesis. The inhibitions of amino acid uptake were the same in the two cases, contrary to a statement made in an initial report of this work (Crathorn & Hunter, 1956). It therefore appears that with these substances the inhibition of the exchange reaction runs parallel with the inhibition of overall protein synthesis.

In experiments with other nitrogen mustards, 'exchange' conditions were normally used, as it was possible to simplify the procedures by using thick suspensions of bacteria in relatively small volumes of buffered solutions.

The series of aromatic nitrogen mustards bearing aliphatic acid side chains was next studied, as in this series there is an interesting variation in the activities of different compounds on the Walker rat carcinoma; this series also contains one of the compounds (M5) that has been used therapeutically. Here a very close correspondence between the activities on the Walker tumour and the amino acid-exchange system was obtained (Table 2). Unfortunately, a supply of the other inactive member with normal alipatic side chains, namely *p*-di-(2-

Table 1. *Comparison of the effectiveness of aliphatic nitrogen mustards in cancer chemotherapy with their ability to reduce the extent of the amino acid uptake in* Staphylococcus aureus *and their toxicity towards this organism*

Nitrogen mustards were used in final concentrations of 6·4 mM. DL-[3-^{14}C]Phenylalanine (specific activity 12 μc/mg.) was added to give a final concentration of 0·32 μc/ml. Under the conditions described in the text, the control cultures incorporated ^{14}C to the extent of approx. 1 μc/g. of protein.

Reference used in text.	Chemical formula of the nitrogen mustards	Effectiveness in cancer chemotherapy	Inhibition of the exchange reaction with phenylalanine (%)	Reduction in viability of the organism under similar conditions (%)
M1	$(CH_3)_2N\cdot CH_2\cdot CH_2Cl$	–	0	0
M2	$H_3C\cdot N(CH_2\cdot CH_2Cl)_2$	+ +	75	50
M3	$N(CH_2\cdot CH_2Cl)_3$	+ + +	98	50

Table 2. *Comparison of the inhibitory effect of acidic aromatic nitrogen mustards on the Walker rat carcinoma with their ability to reduce the extent of amino acid uptake in* Staphylococcus aureus

Nitrogen mustards were added to give a final concentration of 3·3 mM. DL-[3-^{14}C]Phenylalanine (specific activity 12 μc/mg.) was used in a final concentration of 0·32 μc/ml. Under the conditions described in the text, the control cultures incorporated ^{14}C to the extent of approx. 1 μc/g. of protein.

Reference used in text	R, in chemical formula of the nitrogen mustard $(Cl\cdot CH_2\cdot CH_2)_2N\text{-}p\text{-}C_6H_4\cdot R$	Assessment of inhibition of Walker carcinoma	Inhibition of the exchange reaction with phenylalanine (%)
M4	$\cdot CH_2\cdot CO_2H$	+ +	95
M5	$\cdot[CH_2]_3\cdot CO_2H$	+ + +	97
M6	$\cdot[CH_2]_4\cdot CO_2H$	– *	5
M7	$\cdot O\cdot[CH_2]_3\cdot CO_2H$	+	44
M8	$\cdot O\cdot[CH_2]_4\cdot CO_2H$	+	49

* Results of tests were variable. Occasionally found: +.

chloroethyl)aminophenylpropionic acid, was not available. Neither the substituted phenylbutyric acid (M 5) active against the Walker rat carcinoma nor the inactive phenylvaleric acid analogue (M 6) was at all toxic to the bacteria under our conditions and time of exposure, so that here the inhibition of amino acid uptake was even more clearly divorced from the direct toxic effects than with the aliphatic series of nitrogen mustards.

The nitrogen mustard analogue derived from phenylalanine was also examined (Table 3). Here the compound has been resolved (Bergel, Burnop & Stock, 1955*a*), and the different optical isomers exhibit different activities against the Walker rat carcinoma. It was found that amino acid uptake in our system was most effectively inhibited by the L-isomer (M 11). After hydrolysing the compound in 0·05 M-phosphate buffer, pH 7 (KH_2PO_4–NaOH mixture), for 2 days at 37°, no inhibition of the amino acid exchange is observed. It is thus clear that the inhibition is essentially a property of the chloro-compound. It must be stated that considerable variation was found in the percentage inhibition produced by different batches of this nitrogen mustard, and the figures given in Table 3 are representative of the relative values which have always been found.

Finally, a group of aromatic nitrogen mustards bearing amine side chains was tested. These substances have been found to be, in general, less active against the Walker rat carcinoma than those described above, and not many of them showed any appreciable activity in our staphylococcal system. Certain discrepancies are, however, evident from an examination of Table 4, and these are discussed in the next section.

Although most of the measurements of amino acid uptake were made with DL-[3-^{14}C]phenylalanine as the labelled amino acid, on occasion this was replaced by [1-^{14}C]glycine with similar results.

DISCUSSION

Confining our attention, initially, to the results obtained on the bacterial system, we may consider what can be deduced about the primary biochemical action of these mustards.

It might be thought that a significant proportion of the inhibitory effect produced by certain mustards could be caused by chemical interaction of the

Table 3. *Comparison of the effectiveness of optical isomers of* p-*di-*(2-*chloroethyl*)*aminophenylalanine and the hydrolysed* L-*isomer in inhibiting the Walker rat carcinoma with their ability to reduce the extent of amino acid uptake into protein by* Staphylococcus aureus

Nitrogen mustards were added to give a final concentration of 3·3 mM. DL-[3-^{14}C]Phenylalanine (specific activity 12 μc/mg.) was used in a final concentration of 0·32 μc/ml. Under the conditions described in the text, the control cultures incorporated ^{14}C to the extent of approx. 1 μc/g. of protein.

Reference used in text	Isomer of 'phenylalanine nitrogen mustard'	Assessment of inhibition of Walker rat carcinoma	Inhibition of the exchange reaction with phenylalanine (%)
M 9	DL	+ +	56
M 10	D	+	28
M 11	L	+ + +	75
M 12	L (after hydrolysis)	·	10

Table 4. *Comparison of the inhibitory effect of basic aromatic nitrogen mustards on the Walker rat carcinoma with their ability to reduce the extent of the amino acid uptake by* Staphylococcus aureus

Nitrogen mustards were added to give a final concentration of 3·3 mM. DL-[3-^{14}C]Phenylalanine (specific activity 12 μc/mg.) was used in a final concentration of 0·32 μc/ml. Under the conditions described in the text, the control cultures incorporated ^{14}C to the extent of approx. 1 μc/g. of protein.

Reference used in text	R, in chemical formula of the nitrogen mustard $(Cl\cdot CH_2\cdot CH_2)_2N\text{-}p\text{-}C_6H_4\cdot R$	Assessment of inhibition of Walker rat carcinoma	Inhibition of the exchange reaction with phenylalanine (%)
M 13	$\cdot NH_2,HCl$	+(?)	9
M 14	$\cdot CH_2\cdot NH_2,HCl$	–	4
M 15	$\cdot NH\cdot CH_3,HCl$	–	0
M 16	$\cdot N(CH_3)_2,HCl$	+	31
M 17	$\cdot O\cdot[CH_2]_3\cdot N(C_2H_5)_2,HCl$	–	11
M 18	$\cdot N^+(CH_3)_3I^-$	–	42
M 19	$\cdot O\cdot[CH_2]_3\cdot N^+(CH_3)_3Br^-$	–	65

mustards with free amino acid in the medium. Calculations based on the results of chemical experiments (Fruton, Stein & Bergmann, 1946) on the reaction of M2 with phenylalanine showed that under the experimental conditions described above not more than 0·2% of the amino acid could have reacted chemically with the nitrogen mustard, even if direct contact between the two substances had been maintained throughout the period of incubation. Further evidence supporting the view that this effect is negligible is inherent in the results (Table 2) obtained with homologues in the series of aromatic nitrogen mustards having aliphatic acid side chains. Their chemical reactivities, measured in terms of carbonium ion formation, are very similar, but their biological effects measured in the staphylococcal system show great variation.

The interpretation of these results on a chemical basis must therefore take into account the interaction of the nitrogen mustards with the various cellular constituents. There is from these experiments no evidence that the nitrogen mustards actually penetrate the boundaries of the cell. It is clear that such a penetration would be followed by some reaction between the inhibitor and the cellular protein and nucleic acid, but the fact that the toxic effects of the nitrogen mustards are not marked within the short experimental period shows that the extent of the reaction cannot be sufficient to effect any important changes in the overall metabolism of the cells. However, the possibility of a specific reaction of the nitrogen mustards with a site of special importance in protein synthesis cannot be ruled out. If such a site were situated in the cell wall or cytoplasmic membrane, then the transport of amino acids into the cell could clearly be hindered; and the active transport of amino acids across cell walls and membranes must surely be envisaged as a part of the chain of events leading from amino acids to protein. To support this view, the recent work of Cowie & Walton (1956) on amino acid assimilation by *Torulopsis utilis* has shown that, in this micro-organism at least, absorbed amino acids do not exist in the free state at all but enter at once into a metabolic pool where they are associated with sites intimately concerned with protein synthesis. Certainly our results would not preclude an action of the nitrogen mustards on this first stage alone. There is a further possible explanation of our observations: the amino acids in the metabolic pool may exist in local concentrations that are sufficiently high for chemical reaction with the inhibitor molecules to proceed at rates much greater than those pertaining to the extracellular concentrations. If these rates were high enough to prevent the incorporation of a considerable proportion of the amino acid into protein, the differential activities of nitrogen mustards containing chloro-groups of similar chemical reactivities could be ascribed to different extents of penetration into the cell.

It is impossible to say whether the results obtained in this bacterial system can be correlated with results of metabolic studies on higher organisms. If the processes of protein synthesis follow similar pathways in all living cells then our results suggest that, in general, the primary inhibitory action of nitrogen mustards is to produce a block at some stage in the process of protein synthesis. On the assumption that there are certain similarities in the processes of protein synthesis in all organisms, the correlation between the results of tests on the bacterial system and of the same compounds on the Walker rat carcinoma are presented. An additional negative correlation might be mentioned at this point. The compound 1:4-dimesyloxybutane (Myleran) active against chronic myelogenous leukaemia is completely inactive in the bacterial system; nor does it react with amino acids or nucleic acids. Clinical observations, however, suggest that the mode of action is different from that of the nitrogen mustards.

It will be noticed (Table 4) that there are some apparent exceptions to the correlation. In particular it may be significant that the two quaternary compounds (M18 and M19) which showed relatively strong activity in the bacterial system are both very water-soluble. In one case at least the inactivity against the Walker carcinoma was surprising and could probably be explained by rapid excretion of the compound. Very little inhibition of amino acid uptake was found when M13 was examined (Table 4), but it has not been tested against the Walker rat carcinoma except under conditions where other toxic side effects are manifest. Thus the positive reaction that we assign to it must remain in some doubt.

Finally it should be mentioned that alterations appearing in the blood picture in the course of treatment of animals with the aromatic nitrogen mustards with aliphatic acid side chains have recently been described (Elson, 1955). The correlations with our results and with the inhibitions of the Walker rat carcinoma are again close.

SUMMARY

1. An investigation has been made of the effects produced by several aliphatic and aromatic nitrogen mustards on the incorporation of ^{14}C-labelled amino acids into protein by *Staphylococcus aureus*.

2. Different nitrogen mustards inhibited this amino acid incorporation to different extents. Where there was a pronounced inhibition, it could

not be ascribed to a direct toxic effect in any of the cases where this was studied.

3. The compounds that proved to be most effective in reducing the amount of the incorporation of amino acids into the bacterial protein were also, in general, those most effective in inhibiting the growth of the Walker rat carcinoma.

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Some Physicochemical Properties of Human Fibrinogen

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Among the numerous studies of the physicochemical properties of fibrinogen the majority of investigators have confined themselves to the more readily available bovine protein. A preparation of human fibrinogen made by salt fractionation was studied by Holmberg (1944); sedimentation velocity and diffusion measurements gave a molecular weight of 600 000 and a frictional ratio of 3; this leads to an axial ratio of 50, assuming zero solvation. Human fibrinogen prepared by the ethanol fractionation method of Cohn and co-workers had a molecular weight of 400 000 and a frictional ratio of 1·98 from sedimentation-velocity and intrinsic-viscosity determinations, and a molecular weight of 580 000 by osmotic-pressure measurements (Oncley, Scatchard & Brown, 1947). Studies on the double refraction of flow by Edsall, Foster & Scheinberg (1947) led to the conclusion that the fibrinogen molecule could be approximated in shape by a prolate ellipsoid 700 Å long with an axial ratio of 18. The material prepared by further fractionation of fraction '1' (Morrison, Edsall & Miller, 1948) always contained a small proportion of a fast component in both the ultracentrifuge and electrophoresis apparatus. Electrophoretic studies by Avery & Munro (1948) of fractions of human fibrinogen prepared by various salt-fractionation methods showed that only one of the materials prepared by precipitation with phosphate consisted of a single electrophoretic component. In a preliminary investigation of purified human fibrinogen prepared by ether precipitation (Kekwick, Mackay, Nance & Record, 1955) a molecular weight of 340 000 and a frictional ratio of 2·34 were found from sedimentation velocity and diffusion measurements (Caspary & Kekwick, 1954). This gives an axial ratio of 28 on the assumption of an unhydrated prolate ellipsoid. The preparations examined had no additional components either on electrophoresis or in the ultracentrifuge. The present paper deals with more extensive sedimentation and diffusion measurements on similarly purified human fibrinogen. The