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THE EFFECTS OF NITROGEN MUSTARD (HN2) ON ACTIVITIES OF THE PLASMA MEMBRANE OF PC6A MOUSE PLASMACYTOMA CELLS

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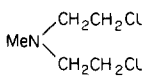
Abstract—Nitrogen mustard, HN2 (10^{-5} M), inhibited the transport of the potassium congener ^{86}rubidium into PC6A mouse plasmacytoma cells by 45% after a 4 hr incubation at 37° *in vitro*. HN2 (10^{-3} M) had a rapid effect on the profile of ^{86}rubidium transport into PC6A cells when added simultaneously with the ^{86}rubidium whereas a monofunctional analogue of HN2((2-chloroethyl)dimethylamine) had no effect at 10^{-3} M. The transport of the amino acid analogues α-aminoisobutyric acid and cycloleucine into PC6A cells was inhibited by 19% and 5% respectively after a 4 hr incubation with 10^{-5} M HN2. The results suggest that the activity of plasma membrane Na^+K^+-ATPase may be affected by HN2. This enzyme may play a pivotal role in controlling cell growth and division. Crude cell membrane preparations from PC6A cells had variable Na^+K^+-ATPase activity which was possibly due to contamination with mitochondrial Mg^{2+}-ATPase. Incubation of a crude cell membrane preparation in the presence of 40 nM dicyclohexylcarbodiimide gave constant Na^+K^+-ATPase activity which was inhibited by 44% on incubation with HN2 (10^{-3} M) for 0.5 hr. The monofunctional analogue of HN2 inhibited this preparation by only 7% under the same conditions. It is suggested that inhibition of Na^+K^+-ATPase by HN2 may be an important facet of its cytotoxic activity.

The mechanism of many antitumour drugs may be multimodal, that is, no single locus of biochemical action may be able to fully explain their cytotoxic effects. This is particularly true for those agents which are capable of covalent reaction with a wide variety of cellular constituents. In such cases it is obviously important to distinguish between those reactions at sites which are causally related to cell death and those which are not, so that any new hypothesis formulated to explain a facet of their mechanism of action should attempt to distinguish between these. This is difficult to achieve and consequently efforts to define the mechanism of action of these drugs, such as the antitumour alkylating agents, continue to be made. Alkylating agents such as nitrogen mustard (HN2, structure I) are generally believed to act by reacting with DNA to prevent cell



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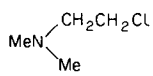

(II)

replication [1]. It is unlikely that, despite some continued claims to the contrary, at physiological concentrations they prevent DNA synthesis [1–5], but cogent evidence has been presented that they crosslink DNA [6], a finding which is compatible with the general requirement for two reactive sites in these drugs (bifunctionality). However, the slowing of DNA synthesis or the presence of crosslinked DNA when cells are in G_2 of the cell cycle cannot as yet fully explain why these drugs are cytotoxic and alternative explanations, or additional sites of action, perhaps complementary to those affecting DNA, may be involved. In this paper we consider loci situated in the cell membrane.

There is considerable current interest in the relationship between changes in the flux of cations and certain aminoacids across the cell membrane and the control of cell replication [7–9]. A mounting body of evidence suggests that sodium ions may play a pivotal role as an intracellular mediator of this control [10–12]. The predictions of Cone [13] that malignant cells would have elevated concentrations of sodium ions have recently been confirmed [14] and this finding suggests that drug induced changes in intracellular ion content may thus be made so as to achieve a selective toxicity. The concentration and activity of both sodium and potassium ions has been shown to change during progression through the cell cycle, changes which have been observed either directly or as reflected by changes in membrane potential [15–17]. These changes occur as the result of alterations in both the cell membrane permeability to selected ions and as the result of changes in the activity of the so-called sodium pump, the enzyme Na^+K^+-ATPase.

It is considered by us that membrane-bound Na^+K^+-ATPase might be a target for bi- or polyfunctional antitumour alkylating agents, like nitrogen mustard. It is sensitive to alkylation as it contains a number of vital-SH groups [18], is a subunit enzyme which can be crosslinked [19] and requires a conformational change for activity [20] which may be

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prevented if subunits are crosslinked. In early studies of the biological properties of the mustards Sir Rudolph Peters suggested that reactions at the cell surface may be important [21] and recently it was shown that HN3, the trifunctional analogue of nitrogen mustard, was preferentially distributed in the cell membrane of human red blood cells, reacting with the membrane components glycophorin and spectrin [22, 23].

Accordingly, we have studied the effects of the bifunctional alkylating agent nitrogen mustard and a monofunctional analogue (structure II) on the transport of ^{86}rubidium ($^{86}Rb^+$) (a potassium congener), α-aminoisobutyric acid and cycloleucine into PC6A cells and have attempted to determine the effects of these agents on the Na^+K^+-ATPase activity of a crude cell membrane preparation from PC6A cells. A preliminary account of this latter part of the work has appeared elsewhere [24].

MATERIALS AND METHODS

Materials. Analytical grade chemicals were obtained from BDH Ltd. (Poole, U.K.) unless otherwise stated. The disodium salt of ATP was obtained free of vanadate ion. Ouabain octahydrate and (2-chloroethyl)dimethylamine were obtained from Sigma Ltd. (Poole, U.K.). Nitrogen mustard was most generously supplied by Dr. V. R. Holland of the Boots Company Ltd. (Nottingham, U.K.). ^{86}Rubidium chloride (1 mCi/224 μg) and cycloleucine (1-aminocyclopentane-1-[^{14}C]carboxylic acid) (59 mCi/mmole) were obtained from the Radiochemical Centre (Amersham, U.K.). [1-^{14}C]Aminoisobutyric acid (53.2 mCi/mmole) was obtained from New England Nuclear (Southampton, U.K.). Media and serum were obtained from Gibco Ltd. (Glasgow, U.K.).

Methods. The ADJ/PC6A plasmacytoma was passaged at seven-day intervals into 20 g female BLAB/c mice by i.p. injection of 0.5 ml of neat ascites. Estimates of the *in vitro* cytotoxicity of nitrogen mustard were obtained by a modified "bioassay" method [25]. 2×10^7 Ascites cells per ml were incubated for 2 hr in RPMI 1640 medium with or without various concentrations of nitrogen mustard, then 1×10^7 cells injected i.p. into groups of five female 20 g BALB/c mice. After 7 days the animals were killed by cervical dislocation and the ascites removed by washing the peritoneum with saline. Inspection of the peritoneal cavity showed that no significant solid tumour deposits had been formed. Cells were counted using a ZB1 Coulter Counter and the approximate log cell kill determined by reference to the numbers of cells harvested from animals which had received serial log dilutions of cells assayed in the same way. In this latter assay a direct relationship between the number of cells injected and the numbers harvested was shown.

Transport studies. Ascites cells were harvested between the sixth and eighth day after transplantation, washed in saline and resuspended at between 5×10^6 to 1×10^7 cells/ml in medium gassed for 1 min with a mixture of 95% air, 5% CO_2. After the required period of incubation with or without drugs, and with continued shaking, isotopes were added: ^{86}rubidium (0.2 μCi/ml), [^{14}C]cycloleucine (1 μCi/ml) or [^{14}C]-α-aminoisobutyric acid (1 μCi/ml). 200 μl Aliquots were removed, in triplicate, at various times from the incubates and placed into tubes containing 100 μl of a mixture of 5 parts of Dow–Corning 550 silicon oil (Hopkins and Williams) and 1.5 parts of corn oil (Mazola) which was layered on top of 50 μl 98% formic acid. The tubes were centrifuged at 9000 *g* for 30 sec (α-aminoisobutyric acid and cycloleucine) or 2 min (^{86}rubidium) using a Beckman microfuge B then frozen in liquid nitrogen and cut through the oil layer. The pellet and supernatant were placed separately into glass vials, thawed and counted. ^{86}Rubidium was counted using an ICN Tracerlab, Gamma Set 500 gamma counter; [^{14}C]cycloleucine and α-aminoisobutyric acid were counted on a Packard Tricarb 2606 scintillation counter using 10 ml NEN 260 scintillant. Preliminary experiments using ^{51}chromium had shown that the density of the oil mixture allowed the passage of 92.4% of the cells through into the formic acid layer but less than 0.2% of the radiolabels from the supernatant. Further experiments showed that the carry over of [^{3}H]inulin in entrapped water was less than 0.3%.

ATPase assays. Two methods were used for the preparation of crude cell membrane fractions of PC6A cells and for estimating the effects of nitrogen mustard upon Na^+K^+-ATPase activity.

Method 1: 3 ml Ascites cells were harvested on the seventh day after transplantation, centrifuged at 300 *g* for 2 min, and the cell pellet resuspended in 7.7 mM $MgCl_2$ in 30 mM Tris–HCl pH 7.4 to a volume of 15 ml. The cell suspension was cooled on ice then sonicated using four 5-sec bursts of optimum power of an Ultrasonic A350g sonicator mounted with a 3 mm titanium probe. This procedure resulted in >98% cell disruption as judged by microscopy. The broken cell suspension was centrifuged at 800 *g* for 10 min at 4° and the pellet resuspended to 30 ml in 30 mM Tris–HCl pH 7.4. 100 μl was incubated at 37° for 15 min with 1.3 ml of either (A) 100 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$ in 30 mM Tris–HCl pH 7.4 or (B) 5 mM $MgCl_2$ in 30 mM Tris–HCl pH 7.4. (The composition of these mixtures had been shown in preliminary experiments to give maximum Na^+K^+-ATPase activity.) Nitrogen mustard or other reagents were then added in 100 μl 30 mM Tris–HCl pH 7.4, which contained a maximum of 20% of either DMSO or ethanol as cosolvent, and the mixture incubated for 0.5 hr at 37° before the addition of 500 μM of disodium ATP. After 10 min at 37° the reaction was terminated by the addition of 2 ml of ice-cold 20% trichloracetic acid, cooled on ice and centrifuged at 500 *g* for 10 min then assayed for inorganic phosphate as described below.

Method 2: 5 ml Ascites, harvested on the 6th to 8th day after transplantation were washed with 0.9% saline in 2 mM HEPES pH 7.4 then resuspended to 20 ml in 2.5 mM HEPES pH 7.4 with 2 mM EDTA at 4°. The suspension was left to stand at 4° for 10 min then sonicated by five 10-sec bursts of optimum power with 30-sec cooling periods. The broken cell preparation was diluted with an equal volume of ice-cold 2.5 mM HEPES pH 7.4, 2 mM EDTA and centrifuged at 10,000 *g* for 10 min at 4°. The

pellet was resuspended in 20 mM HEPES pH 7.4 to give a protein concentration of between 2 and 5 mg/ml. Drugs or reagents were then added either in buffer or in 200 μl of the suspension and the mixture incubated for 0.5 hr at 37° with occasional mixing before centrifuging at 27,000 *g* for 15 min at 4°. The pellet was resuspended in the minimal volume (400–800 μl) of 20 mM HEPES pH 7.4 containing 10 mM $MgCl_2$ and 5 mM EGTA and 150 μl added to 850 μl of solution (A) or (B) (see method 1 above) with the substitution of 20 mM HEPES pH 7.4 for Tris–HCl. After a 5-min preincubation period with or without 40 nM dicyclohexylcarbodiimide, 500 μM of disodium ATP was added and after 10 min at 37° the reaction was terminated by the addition of 1.5 ml of ice-cold 20% trichloracetic acid. After cooling on ice the mixtures were centrifuged at 500 *g* for 10 min and assayed for inorganic phosphate as described below. Protein concentrations were determined by the method of Lowry *et al.* [26] using bovine serum albumin as a standard.

Assay of inorganic phosphate. The method was a modification of that of Baginski *et al.* [27]. 1 ml of the assay mixtures described above was mixed sequentially with 1 ml 4% ascorbic acid in a solution of 1 mg/ml EDTA, 0.5 ml 2% ammonium molybdate and 2 ml of a mixture of 2% trisodium citrate and 2% sodium arsenite. The blue colour was allowed to develop for 15 min and the absorbance read at 700 nm. The assay was linear for up to 400 μM inorganic phosphate.

RESULTS

Estimation of the cytoxicity of HN2 to PC6A cells in vitro. The results of the *in vitro–in vivo* bioassay are shown in Fig. 1. A greater than 99% cell kill was observed with a concentration of approximately 10^{-5} M HN2. This concentration was subsequently used for most of the studies outlined below. HN2 has a chemotherapeutic index of 4.4 against the PC6A *in vivo* with an ID_{90} of 0.8 mg/kg, which, assuming equal distribution, gives 4×10^{-6} M, thus

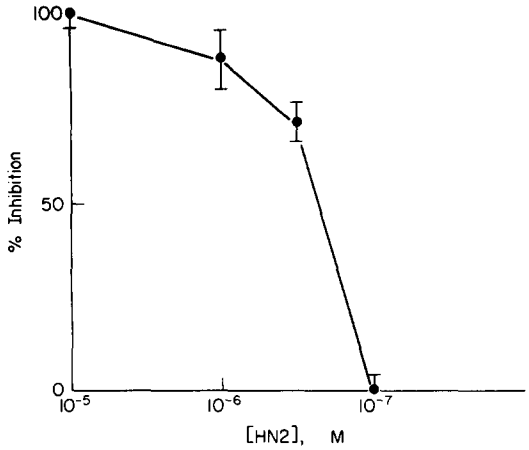

Fig. 1. Results of the *in vitro–in vivo* bioassay of PC6A cells with HN2. ($n = 4$, ±S.E.M.)

10^{-5} may be a slight overestimate of a 'physiologically relevant' concentration.

Transport studies. The rate of transport of $^{86}Rb^+$ into PC6A cells, expressed as the fraction of counts taken up into the cells divided by the counts in the supernatant was observed to be linear for the first 10 min but thereafter was curvilinear presumably as the rate of efflux became significant (Figs. 2 and 3). Nitrogen mustard at high concentrations (10^{-2}, 10^{-3} M) had a profound effect on the pattern of $^{86}Rb^+$ accumulation (Fig. 2(A)) whereas a monofunctional analogue of nitrogen mustard, which cannot crosslink cellular constituents, had no effect (Fig. 2(B)). The lack of effect of lower concentrations of nitrogen mustard (e.g., 10^{-5} M) when added simultaneously with $^{86}Rb^+$ to PC6A cells may be explained by the slow rate of inactivation of an essential component. After longer periods of incubation their effects were observed to be profound (Figs. 3 and 4).

The effect of 10^{-5} M nitrogen mustard on the transport of the amino acid analogue α-aminoisobutyric

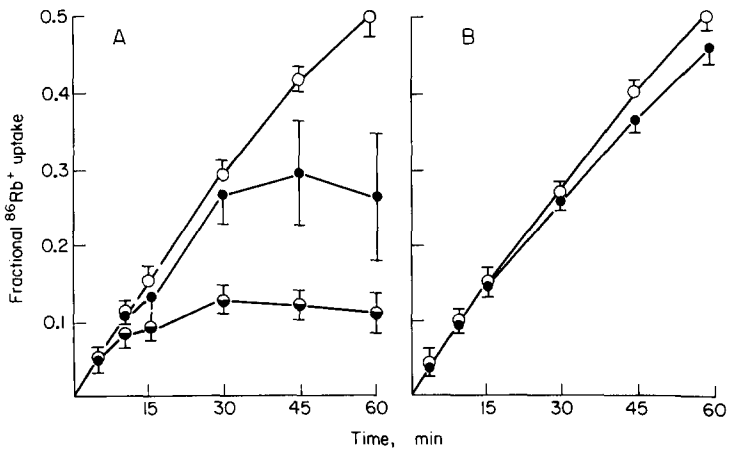

Fig. 2. The effects of HN2 (A) and (2-chloroethyl)dimethylamine (B) on the fractional uptake of rubidium into PC6A cells with time. (A) ○ = control; ● = 10^{-3} M HN2; ◒ = 10^{-2} M HN2, each added at time = 0. (B) ○ = control; ● = 10^{-3} M (2-chloroethyl)dimethylamine added at time = 0. ($n = 3$, ± S.E.M.)

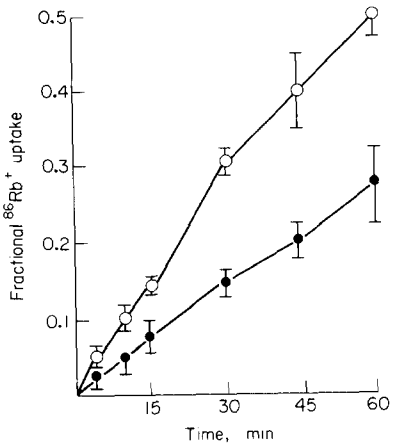

Fig. 3. The effect of a 4 hr incubation with HN2 (10^{-5} M) on the fractional uptake of rubidium into PC6A cells. ○ = control; ● = HN2 (10^{-5} M). (n = 3, ±S.E.M.)

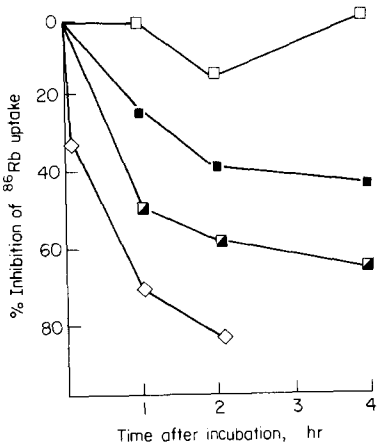

Fig. 4. Percentage inhibition of the fractional rubidium uptake into PC6A cells after timed incubations with various concentrations of HN2. Results are expressed as a percentage of the uptakes observed after 10 min of rubidium uptake. ◇ = 10^{-3} M HN2; ◪ = 10^{-4} M HN2; ■ = 10^{-5} M HN2; □ = 10^{-6} M HN2.

acid (αAIB) into PC6A cells was less marked than the effect on $^{86}Rb^+$. The level of the total uptake of αAIB was much more variable than that of $^{86}Rb^+$ so that Fig. 5(A) shows a representative result rather than mean results. The mean % inhibition of αAIB uptake, at 10 min was 18.8 ± 2.7% (n = 5). Whereas the transport of αAIB appeared to be linear for several minutes that of cycloleucine was linear only for 90 sec (Fig. 5(B)) a result similar to that seen using other cell types [28]. Under identical conditions to those in which $^{86}Rb^+$ and αAIB uptake were inhibited by a 4 hr incubation with 10^{-5} M nitrogen mustard there appeared to be little effect on cycloleucine transport with a mean inhibition of 5.2 ± 1.4% at 90 sec (n = 3).

ATPase inhibition. In a preliminary communication we reported that HN2 was a potent inhibitor of Na^+K^+-ATPase activity in a crude cell membrane preparation [24]. The preparation used (method 1, above) was based on the method of Taylor [29]. Continued study of this preparation has, however, shown it to be extremely variable: Na^+K^+-ATPase activity, as defined by activity in solution A minus B (see Materials and Methods) was 2.2 ± 0.9 μmoles inorganic phosphate (P_i)/mg protein/hr (n = 8, ± S.E.M.) with certain preparations having negligible activity. This raised certain doubts about the preliminary study of inhibition by HN2 (although similarly high potencies of inhibition had been reported for adriamycin [30]) and subsequently alternative preparations and protocols were investigated.

Method 2 (see Materials and Methods) gave a total ATPase activity of 2.1 ± 0.14 μmoles P_i/mg/hr (n = 8). The addition to the assay of the selective mitochondrial Mg^{2+}-ATPase inhibitor dicyclohexylcarbodiimide (DCCD) [32] reduced total ATPase activity to 1.7 ± 0.12 μmoles P_i/mg/hr (n = 8) which strongly suggests that mitochondrial contamination was the reason for the variability. Consequently, all assays were then conducted in the presence of 40 nM DCCD, a concentration which was found to allow an optimum Na^+K^+-ATPase activity of 0.54 ± 0.03 μmoles P_i/mg/hr (n = 11)

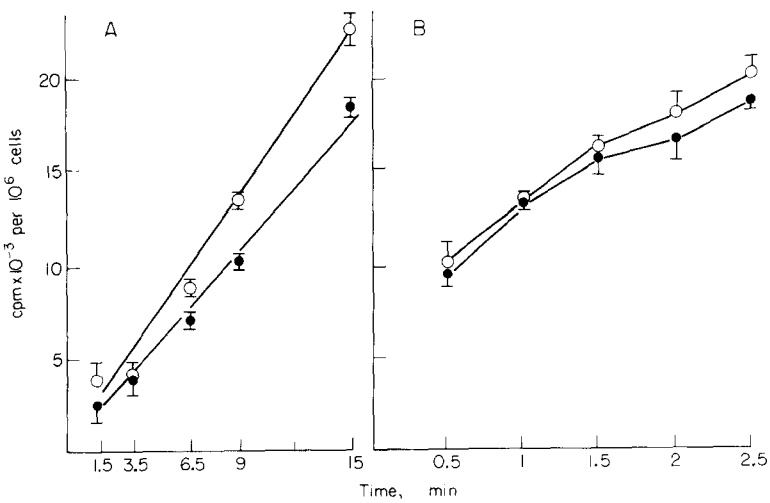

Fig. 5. The effect of HN2 (10^{-5} M) on the uptake of (A) α-aminoisobutyric acid; (B) cycloleucine into PC6A cells after 4 hr incubation at 37°. ○ = controls; ● = treated.

which was inhibited 91 ± 7% ($n = 3$) by 10^{-3} M ouabain.

Incubation of crude cell membrane preparations with HN2 (10^{-3} M) for 0.5 hr at 37° caused a 43.5 ± 7.1% ($n = 4$) inhibition of Na^+K^+-ATPase activity whilst the non sodium–potassium dependent ATPase activity (total ATPase–Na^+K^+-ATPase activity) was inhibited by only 8.7 ± 4.0% ($n = 3$) suggesting that HN2 was a specific inhibitor of Na^+K^+-ATPase under these conditions. Under identical conditions the monofunctional analogue of HN2 ((2-chloroethyl)dimethylamine, II) caused only a 6.5 ± 7.1% inhibition of Na^+K^+-ATPase ($n = 4$).

DISCUSSION

The results presented here show that the bifunctional alkylating agent nitrogen mustard (I) has a considerable inhibitory action on certain aspects of the membrane function of PC6A cells treated *in vitro*, whereas under identical conditions a monofunctional analogue (II) does not. In particular it appears that the mechanisms of membrane transport considered to be associated with the existence of a sodium gradient [33] and thus the activity of the enzyme Na^+K^+-ATPase, are affected since the transport of α-aminoisobutyric acid was inhibited (Fig. 5(A)) whereas that of cycloleucine, considered to be sodium-independent, was not (Fig. 5(B)). The radiomimetic characteristics of nitrogen mustard are possibly a consequence of this action, amongst others, since similar observations of the inhibition of sodium-dependent, neutral amino-acid transport have been observed after the γ-irradiation of a cultured human T-Cell line [34]. Sulfhydryl groupings in the membrane, which are proximal to each other, have been implicated in the mechanism of transport of neutral amino acids, like α-aminoisobutyric acid [35], and it thus might be expected that this process would be sensitive to an alkylating agent.

The effects of a low concentration of HN2 on transport seen here, particularly on $^{86}Rb^+$ (Fig. 4) are comparable, in both timing and the extent of inhibition, to the well-documented effect of alkylating agents upon thymidine incorporation into DNA, sometimes taken as a presumptive measure of DNA synthesis. This effect on thymidine, in the absence of similar effects on the incorporation of precursors of RNA and protein synthesis, has been used in argument to suggest that DNA is thus the primary target for alkylating agent activity [36]. It may thus be similarly argued that the membrane is of comparable importance, although it is possible that the effects of certain alkylating agents on thymidine are in any case related to an action at the membrane [3].

The ineffectiveness of a monofunctional analogue of nitrogen mustard (II) to inhibit the transport of $^{86}Rb^+$ into PC6A cells (Fig. 2(B)) suggests that bifunctionality, and the ability to cross-link a target in the membrane, may be an important aspect of the action of HN2. The crosslinking of membrane components by chloroethylamine alkylating agents has been demonstrated by others mainly to affect structural aspects of membrane function [22, 23, 37]. However, as mentioned above, Na^+K^+-ATPase is crosslinked and inactivated by agents such as copper phenanthroline [38] by a mechanism which appears to involve proximal sulfhydryl groupings [20] and it is possible to speculate that a similar effect may occur on covalent reaction of the enzyme with HN2.

This supposition was partially confirmed by comparing the effects of HN2 and the monofunctional analogue on the Na^+K^+-ATPase activity of crude cell membrane preparations (see Results). Problems with the variability in enzyme activity, although partially resolved by the inhibitory effect of DCCD on possible mitochondrial ATPase contamination, suggest that further studies on the potency and mechanism of alkylating agent inhibition will be best made on a purified preparation.

Enzymes have previously been considered as secondary targets for the effects of antitumour alkylating agents as it has been argued that the number of enzyme molecules was too great in comparison to the molarity of alkylating agent likely to be present under physiological conditions [39]. This assumes that distribution of the alkylating agent is likely to be uniform whereas in the case of highly reactive agents, such as HN2, it might be assumed that a gradient exists of alkylated products which is maximal at the outer extremities of the cell. In the study of the distribution of [^{14}C]-HN3 this was confirmed [22].

Similar results to those described above have recently been reported in studies on the mechanism of action of the aziridinylbenzoquinone Trenimon [40].

It remains to establish whether there is a causal relationship between the effects described here (and by others [40]) and consequent cell death. A gross imbalance of intracellular ion content would be expected to have profound effects on cellular metabolism, including those processes in which subtle changes in ion concentration appear to play a major role in the control of cellular growth and division and in the integrity of chromosomal material.

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