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ACTIVATION IN VITRO OF NITROGEN MUSTARD BY LIPOSOMAL TRANSPORT

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We have already reported that macromolecule synthesis in Ehrlich cell mitochondria is strongly inhibited by chemotherapeutic doses of the alkylating agent nitrogen mustard (HN2), when RNA and protein synthesis are measured in either the intact cells, or in the mitochondria isolated from these cells after HN2 treatment (1). However, even at high concentrations isolated mitochondria themselves are resistant to HN2. Table 1 shows that a concentration of 2.5 x 10^{-3} M had no significant effect on RNA or protein synthesis, whereas a dose *in vivo* of as little as 0.4 mg/kg, produced a 40 per cent inhibition of protein and RNA synthesis. A dose of 0.4 mg/kg is equivalent to a maximum intraperitoneal concentration of $\sim 10^{-5}$M and a maximum intracellular concentration of 5 x 10^{-4} M (2,3).

TABLE 1. Comparative effects *in vivo* and *in vitro* of nitrogen mustard on RNA and protein synthesis

HN2 (mg/kg)	HN2 [M]	% of Control Incorporation			
		In vivo*		In vitro†	
		Protein	RNA	Protein	RNA
0.4	1.00×10^{-5}	61	60	--	--
1.0	2.50×10^{-5}	63	59	--	--
5.0	1.25×10^{-4}	44	48	98	101
---	2.50×10^{-3}	--	--	93	95

* *In vivo* - HN2 was administered i.p. After 30 min ascites cells were removed and mitochondria prepared according to Avadhani *et al.* (1). Protein (10-12 mg) was incubated at 35° in 25 mM Tris-HCl (pH 7.5), 40 mM KCl, 10 mM KH_2PO_4, 7 mM Mg $(CH_3COO^-)_2$, 5 mM 2-mercaptoethanol and 0.25 M sucrose

containing 10 μCi ^{3}H-leucine (sp. act. ~ 20 Ci/m-mole) or ^{3}H-cytidine (25 Ci/m-mole), 6 μmoles/ml phosphoenolpyruvate, 3 μmoles/ml each of ATP and GTP, and 4 μg/ml of pyruvate kinase. For protein synthesis, 100 μg/ml each of emetine and cycloheximide were included. Protein was precipitated with trichloracetic acid and assayed by scintillation counting. The control value for protein synthesis was 9.9×10^{3} cpm/mg of protein/30 min, and for RNA synthesis, 18.6×10^{3} cpm/mg of protein/60 min.

$^{+}$In vitro - Mitochondria were prepared from untreated cells as in (*) above and preincubated with HN2 for 30 min in the basic 0.25 M sucrose medium. Then ^{3}H-leucine or ^{3}H-cytidine, enzymes and cofactors were added and the incubation was continued for 30 min for protein and 60 min for RNA. Appropriate control values are: protein, 8.8×10^{3} cpm/mg, and RNA, 14.1×10^{3} cpm/mg of protein.

The striking contrast between mitochondria exposed to HN2 in vivo and in vitro suggested a difference in permeability to HN2 under the two conditions. We therefore decided to test the effect of a hydrophobic carrier on HN2 action and selected simple phospholipid vesicles (liposomes) (4,5) for this purpose, since these vesicles are known to carry hydrophobic drugs on their surface, within their lipid double layer, or inside the vesicle proper (6-8). Sonicates were prepared using 10 mg/ml of L-α-lecithin in 0.9% NaCl. These sonicates were mixed with equimolar concentrations of HN2, based on phospholipid P. The effects of the drug-phospholipid (HN2-PL) combination on RNA and protein synthesis are presented in Figs. 1 and 2 respectively. In both cases, 10^{-5} M HN2-PL produced an almost complete inhibition of biosynthetic activity. Our currently available data indicate that the 50 per cent inhibition index for HN2-PL lies between 5×10^{-7} and 10^{-6} M, but we have not yet completed detailed titrations. The activity of HN2-PL against isolated mitochondria is several orders of magnitude greater than that of HN2 alone in vitro and between 10 and 100 times as great as the effects in vivo of the drug.

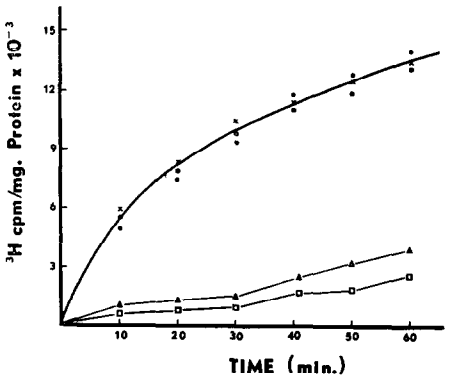

Fig. 1

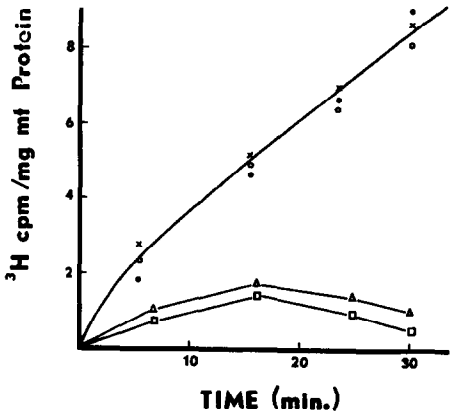

Fig. 2

Fig. 1. RNA synthesis in isolated mitochondria. Conditions as listed in Table 1. ^{3}H-cytidine incorporation into RNA after treatment with phospholipid (PL) or nitrogen mustard-phospholipid mixture HN2-PL. —○— Control; —●— nmoles PL/ml; —✱— 100 nmoles PL/ml; —△— nmoles HN2-PL/ml; —□— 100 nmoles HN2-PL/ml.

Fig. 2. Protein synthesis in isolated mitochondria. Conditions listed in Table 1, symbols as in Fig. 1. ^{3}H-leucine incorporation into protein after treatment with PL or HN2-PL.

As yet we have no explanation of whether the phospholipid effects on HN2 action are simply a reflection of non-specific permeability changes or are specific transport effects. Experiments using a 0.05 to 0.075% non-ionic detergent (NP-40) show 50-60 per cent increases in RNA and protein synthesis but no stimulation of HN2-inhibitory action *in vitro*. Since the phospholipid concentration used is < 0.01 per cent, it is unlikely that a non-specific detergent effect is involved. Phospholipids present in liposomes are known to fuse with cell membranes as well as to traverse the membranes, enter the interior of cells and fuse with lysosomes (6-9). Thus, a direct localization by fusion with the outer mitochondrial membrane is conceivable, although there are no previous reports of fusion of liposomes *in vivo* or *in vitro* with mitochondria (C. D. deDuve, personal communication). If it is assumed that 10 per cent of the mitochondrial phospholipid is present in its outer membrane, exclusive incorporation of the added phospholipid into this membrane on random basis would effect only 1 in a 1000 sites. We are currently examining specific permeability changes, since evidence of the potentiation *in vivo* of drug action by liposomes (10,11) makes this an attractive possibility.

REFERENCES

1. N. G. Avadhani, G. Hansel, C. Ritter and R. J. Rutman, Cancer Biochem. Biophys. in press.

2. R. J. Rutman, E. H. L. Chun and F. S. Lewis, Biochem. biophys. Res. Commun. 32, 650 (1968).

3. E. H. L. Chun, L. Gonzales, F. S. Lewis, J. Jones and R. J. Rutman, Cancer Res. 29, 1184 (1963).

4. G. Sessa and G. Weissman, J. Lipid Res. 9, 310 (1968).

5. A. D. Bangham, A. Rev. Biochem. 43, 753 (1972).

6. G. Gregoriadis, Fedn Eur. Biochem. Soc. Lett. 36, 292 (1973).

7. D. Papahadopoulous, G. Poste and E. Mayhew, Biochim. biophys. Acta 363, 404 (1974).

8. Y. Rahman and B. J. Wright, J. Cell Biol. 65, 112 (1975).

9. D. Papahadjopoulous, E. Mayhew, G. Poste, S. Smith and W. J. Vail, Nature, Lond. 252, 163 (1974).

10. G. Gregoriadia and E. D. Neerunjun, Res. Commun. Chem. Path. Pharmac. 10, 351 (1975).

11. R. J. Rutman, N. G. Avadhani and C. Ritter, Cancer Chemother. Rep., in press.