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The DPN content of mitochondria isolated from ascites-tumour cells treated with a carcinostatic ethyleneimine

Carcinostatic ethyleneimines inhibit the glycolysis of tumours under conditions in which respiration is little affected[1,2]. This inhibition of glycolysis has been shown to be due to a marked decrease in the DPN^+ content of the cells, probably brought

TABLE I

DPN^+ CONTENT OF ASCITES CELLS AND TOTAL DPN CONTENT OF ASCITES-CELL MITOCHONDRIA ISOLATED FROM TRENIMON-TREATED AND CONTROL CELLS

Ehrlich ascites cells from 15–20 mice were collected in 100 ml 0.9% NaCl plus 1 mM EDTA (pH 7) and washed free from ascites serum and erythrocytes by centrifuging 2 or 3 times at low speed at room temperature in Ringer solution. The cells were then taken up to 250 ml in Ringer–bicarbonate buffer which had been equilibrated with 5% CO_2–95% O_2 (final pH 7.4) and distributed between four 300-ml erlenmeyers (4 times 60 ml) and shaken in a Warburg bath at 37° under continuous gassing. After 10–15 min, 5 ml of a freshly prepared Trenimon solution (1.0 g/l Ringer–bicarbonate in Expt. 3 and 0.5 g/l in other experiments) and 0.6 ml 40% glucose were rapidly added to erlenmeyers 1 and 2, while 6 ml Ringer–bicarbonate and 0.6 ml 40% glucose were added to erlenmeyers 3 and 4. The incubation was continued under constant gassing until definite inhibition of aerobic glycolysis, which was measured semi-quantitatively by manometry, was observed. The cells were then rapidly cooled to 0° and left at that temperature during all subsequent manipulations. Trenimon-treated and control cells were treated identically and, where possible, handled simultaneously. The cells were centrifuged at high speed, taken up in two lots of 45 ml 0.25 M sucrose, and resuspended by hand with the aid of a Potter–Elvehjem homogenizer. Samples (3 ml) were removed for DPN^+ determination and kept in ice. The cells were recentrifuged at high speed and after discarding the supernatant, mitochondria were prepared in 0.25 M sucrose[6], using the Servall automatic refrigerated centrifuge for differential centrifugation. At the moment that the cells were homogenized the corresponding sample for DPN^+ determination was deproteinized by adding 0.5 ml 40% trichloroacetic acid. In Expts. 2, 3 and 4 the mitochondrial preparation was incubated with 0.2 mM 2,4-dinitrophenol, 5 mM nicotinamide and 0.01 M phosphate buffer (pH 7.4) for 5 min at room temperature to convert the DPNH present in the preparation into DPN^+ (*cf.* ref. 7) after which 40% trichloroacetic acid was added to a final concentration of 4%. In Expt. 1 this preincubation was omitted and the trichloroacetic acid was added at once. DPN^+ was determined in the neutralized acid extracts in either of two ways: In the cell extracts DPN^+ was determined in duplicate in a reaction mixture containing 4% ethanol and 0.1 M Tris–acetate buffer (pH 10.1). The DPN^+ content of the sample was calculated from the absorbancy change after addition of yeast alcohol dehydrogenase (EC 1.1.1.1), measured with an Eppendorf photometer at 366 mμ. In the mitochondrial extracts of Expts. 3 and 4 the same procedure was followed but readings were made on a Zeiss spectrophotometer at 340 mμ. In Expts. 1 and 2 the DPN^+ content of the mitochondrial extracts was determined by an enzymic–fluorometric method[5]. Readings were made on the Eppendorf photometer with fluorometric attachment. Protein was determined by the biuret method with egg albumin as standard[5]. Packed cell volumes were estimated with a Kafka tube. Trenimon was obtained from Bayer A.G.

	Trenimon present	*Expt.*			
		1	*2*	*3*	*4*
Incubation with Trenimon (min)		*53*	*65*	*50*	*38*
Whole cells:					
DPN^+ (μmoles/ml packed cells)	—	0.20	0.23	0.13	0.20
DPN^+ (μmoles/g protein)	—	2.66	1.74	1.14	1.73
DPN^+ (μmoles/g protein)	+	0.29	0.15	0.06	0.44
Change (%)		−89	−91	−95	−75
Mitochondria:					
DPN (μmoles/g protein)	—	1.8	2.2	4.2	6.2
DPN (μmoles/g protein)	+	1.9	2.1	2.9	5.3
Change (%)		+6	−4	−31	−14

Abbreviation: Trenimon, 2,3,5-tris(ethyleneimino)-1,4-benzoquinone.

about by an inhibition of DPN synthesis[3]. The fact that respiration is not inhibited could be explained in two ways:

(a) The oxidation of endogenous substrates (mainly fat) requires very little DPN.

(b) The decrease in cellular DPN^+ is due to a decrease of extramitochondrial DPN^+, while the mitochondrial DPN^+ is little affected[4]. This would imply that the membrane of ascites-tumour mitochondria is relatively impermeable to DPN not only *in vitro*, as demonstrated previously[5], but in the intact cell as well.

Support for the second possibility has now been obtained in experiments summarized in Table I. Ascites cells were aerobically incubated in Ringer–bicarbonate in the presence of Trenimon, a new ethyleneimine which has an effect on tumours similar to that of the other ethyleneimines studied in this laboratory (for a summary, see ref. 8). After inhibition of glycolysis had set in, the DPN^+ content of the cells and the total DPN content of mitochondria isolated from them were determined and compared with corresponding values of untreated controls. It is clear from the results that the mitochondrial DPN content is little affected even though the DPN^+ content of the whole cells decreases 75–95 %.

10–15 % of the total DPN present in ascites cells is located in the mitochondria (*cf.* refs. 9 and 10). Thus, a 90 % decrease in the total DPN content of ascites cells is equal to a complete disappearance of DPN from the extra-mitochondrial compartment. Whether this has actually been reached in our experiments cannot be calculated since we have measured only DPN^+, and not the total DPN in the whole cells. However, an estimate can be made from the few observations reported in the literature. In ascites cells incubated anaerobically with A 139 [2,5-bis(methoxyethoxy)-3,6-bis(ethyleneimino)-1,4-benzoquinone] SCRIBA[11] found a decrease of 71 % in the total DPN content while the DPN^+ content decreased 80 %. In addition HILZ *et al.*[12] mention that the fall in DPN^+ content after irradiation of ascites cells is also accompanied by a fall in the DPNH content. It seems probable therefore that in our Expts. 1–3 practically all the DPN^+ left in the cells after incubation with Trenimon must have been in the mitochondria.

Our results provide an explanation for the biphasic curve obtained by HILZ *et al.*[12] for the loss of DPN^+ in ascites cells treated with Trenimon under aerobic conditions. Within the first 20 min, 80–85 % of the DPN is lost and the subsequent loss of DPN^+ proceeds slowly at a rate less than one-tenth of that observed in the first 20 min. It seems reasonable to conclude that the rapid phase represents the loss of DPN^+ from the extra-mitochondrial compartment while the slow phase represents the slow leakage of DPN^+ from the mitochondria. If this interpretation is correct a more extensive analysis of the slow phase of DPN loss might give some information on the rate of turnover of mitochondrial DPN in intact ascites cells.

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[1] I. M. ROITT, *Biochem. J.*, 63 (1956) 300.
[2] H. HOLZER, G. SEDLMAYR AND A. KEMNITZ, *Biochem. Z.*, 328 (1956) 163.
[3] H. KRÖGER, H. W. ROTTHAUWE, B. ULRICH AND H. HOLZER, *Biochem. Z.*, 333 (1960) 148; 333 (1960) 155.
[4] P. BORST, *Symp. on Functional Biochemistry of Cell Structures, Proc. Vth Intern. Congr. Biochem., Moscow 1961*, Vol. 2, Pergamon Press, Oxford, in the press.
[5] P. BORST AND J. P. COLPA-BOONSTRA, *Biochim. Biophys. Acta*, 56 (1962) 216.
[6] P. BORST, *11th Yearbook for Cancer Research and Fight against Cancer in the Netherlands*, 1961, p. 227.
[7] E. C. SLATER, M. J. BAILIE AND J. BOUMAN, *Proc. Symp. on Biological Structure and Function, Stockholm, 1960*, Vol. 2, Acad. Press, London, p. 207.
[8] H. KRÖGER, B. ULRICH AND H. HOLZER, *Arzneimittelforsch.*, 9 (1959) 598.
[9] B. HESS, *Proc. Symp. on Funktionelle und morphologische Organisation der Zelle, Rottach-Egern, 1963*, Springer Verlag, Heidelberg, p. 163.
[10] P. BORST, *Proc. Symp. on Funktionelle und morphologische Organisation der Zelle, Rottach-Egern, 1963*, Springer Verlag, Heidelberg, p. 137.
[11] P. SCRIBA, *Thesis*, University of Freiburg i.Br., 1959.
[12] H. HILZ, B. HUBMANN, M. OLDEKOP, M. SCHOLZ AND M. V. GOSSLER, *Biochem. Z.*, 336 (1962) 62.

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