6. D. G. ATTARDI, R. LEVI-MONTALGINI, B. S. WENGER and P. U. ANGELETTI, *Science* **150,** 1307 (1965).
7. D. G. ATTARDI, M. J. SCHLESINGER and S. SCHLESINGER, *Science* **156,** 1253 (1967).
8. I. SCHENKEIN, M. LEVY, E. D. BUEKER and E. TOKARSKY, *Science* **159,** 640 (1968).
9. I. SCHENKEIN, M. BOESMAN, E. TOKARSKY, L. FISHMAN and M. LEVY, *Biochem. biophys. Res. Commun.* **36,** 156 (1969).
10. T. O. EKFORS, T. MALMIHARJU and V. K. HOPSU-HAVU, *Enzymologia* in press (1972).
11. T. O. EKFORS and V. K. HOPSU-HAVU, *Enzymologia* in press (1971).
12. E. WERLE and P. RODEN, *Biochem. Z.* **286,** 213 (1936).
13. E. WERLE, R. VOGEL and J. LENTRODT, *Archs int. Pharmacodyn. Ther.* **126,** 187 (1960).
14. S. COHEN, *Proc. natn. Acad. Sci.* **46,** 302 (1960).
15. R. LEVI-MONTALGINI and S. COHEN, *Ann. N.Y. Acad. Sci.* **85,** 324 (1960).
16. N. BACK, *Fedn. Proc.* **25,** 77 (1966).
17. U. HAMBERG, *Proc. Roy. Soc. B.* **173,** 393 (1969).
18. R. W. COLMAN, *Biochem. biophys. Res. Commun.* **35,** 273 (1969).
19. K. D. BHOOLA, J. D. CALLE and M. SCHACHTER, *J. Physiol., Lond.* **152,** 75 (1960).
20. T. ASTRUP and S. MÜLLERTZ, *Archs Biochem. Biophys.* **40,** 346 (1952).
21. P. BRAKMAN, *Fibrinolysis, A Standardized Fibrin Plate Method and a Fibrinolytic Assay of Plasminogen.* Sheltema & Holkema, Amsterdam (1967).
22. T. O. EKFORS, V. K. HOPSU-HAVU and T. MALMIHARJU, *Acta physiol. scand.* **75,** 157 (1969).
23. R. VOGEL, *Hoppe Seyler's Z. Physiol. Chem.* **349,** 930 (1968).
24. I. TRAUTSCHOLD, *Enzyme und Inhibitoren der Bildung und des Abbaues gefässaktiver Polypeptide* (Kinine). Habilitationsschrift der medizinischen Fakultät München (1965).
25. K. D. BHOOLA, *J. Physiol., Lond.* **196,** 431 (1968).
26. K. D. BHOOLA, *Biochem. Pharmac.* **18,** 1252 (1969).
27. T. O. EKFORS and V. K. HOPSU-HAVU, *Histochem. J.* in press (1972).
28. S. Y. ALI and L. EVANS, *Biochem. J.* **107,** 293 (1968).
29. W. TROLL and S. SHERRY, *J. biol. Chem.* **213,** 881 (1955).
30. D. L. KLINE and J. B. FISHMAN, *J. biol. Chem.* **236,** 2807 (1961).
31. K. O. KJELDGAARD and J. PLOUG, *Biochim. biophys. Acta* **24,** 283 (1957).
32. S. SHERRY, N. ALKJAERSIG and A. P. FLETCHER, *J. Lab. clin. Med.* **64,** 145 (1964).
33. P. L. WALTON, *Biochim. biophys. Acta* **132,** 104 (1955).

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Observations on the metabolism of cyclophosphamide

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CYCLOPHOSPHAMIDE (Cytoxan, Endoxan, *N*, *N*-di-2-chloroethyl-*N'O*-trimethylenephosphordiamide[1]), is one of the safest and most widely used agents in the treatment of many cancers. Although originally designed to be activated by tumour cells possessing high levels of a phosphoramidase enzyme[2] it is now well established that cyclophosphamide is activated almost exclusively by the liver. Since this fact was recognised, widespread efforts have been made to isolate and identify the active metabolite. Although metabolites have been isolated from serum[3] and urine[4] and following treatment with liver preparations[5] which activate cyclophosphamide *in vitro*, there is no unequivocal evidence that any one of these compounds is the active cytotoxic metabolite.

We have developed a bioassay method whereby the formation of cytotoxic derivatives of cyclophosphamide in the presence of liver microsomal preparations and an NADPH-generating system can be assayed by incubation with tumour cells.[6] Thereafter aliquots of the incubates, containing known amounts of tumour cells, are injected intraperitoneally into recipient rats. Comparison of the survival times of these animals with those of control animals, receiving an inoculum of tumour cells incubated with cyclophosphamide in the absence of a microsomal activation system, gives a reasonably precise measure of the cytotoxicity of the metabolite formed.

When radioactively labelled [^{32}P]cyclophosphamide was used and the incubation mixture extracted with chloroform (efficiency 80 per cent) the radioactive compounds present in the extract were detected

by a combination of thin-layer chromatography (TLC) and scanning for radioactivity. The rate of formation of the metabolites could be correlated with the level of cytotoxicity towards tumour cells.

Using this assay system the following points have been established.

(a) Incubation of cyclophosphamide with rat liver microsomes and an NADPH-generating system produces a metabolite which is as toxic *in vitro* to Walker 256 tumour cells as cyclophosphamide is to these cells *in vivo*. Microsomes alone are incapable of producing this activation and NADH cannot replace NADPH.

(b) The active metabolite is relatively unstable, decomposing in the incubation mixture apparently with the formation of an ionic phosphorus-containing substance. Although the metabolite is highly toxic to tumour cells the breakdown products are non-toxic.

Figure 1 shows that when tumour cells were incubated with microsomes, NADPH, and cyclophosphamide (60 μg/ml), cytotoxicity towards the tumour cells increased up to about 1 hr, after which time there was no further increase in toxicity. In previous reports, where colorimetric methods were used to estimate metabolism, it was shown that microsomal preparations activated cyclophosphamide linearly up to 20–45 min.[7,8] Therefore, incubations of cells, drug and activating systems were usually carried out for 45 min.

When ^{32}P-labelled cyclophosphamide (60 μg/ml) was incubated with tumour cells alone or with washed microsomes or with microsomes and NADH, the only major radioactive product in the chloroform extract of the incubate had an R_f value in TLC identical with that of authentic cyclophos-

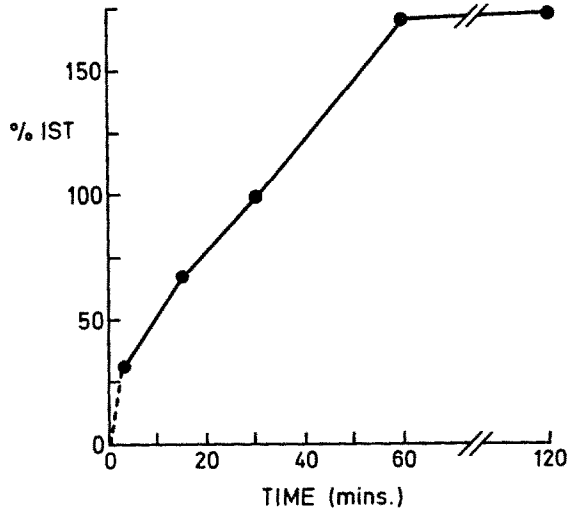

FIG. 1. Tumour cells (10^6/ml) were incubated in nutrient fluid buffered at pH 7·4 in the presence of microsomes, NADPH-generating system (glucose-6-phosphate, NADP, $MgCl_2$, nicotinamide and glucose-6-phosphate dehydrogenase), and cyclophosphamide (60 μg/ml). At intervals, aliquots of the cells were removed and injected into recipient rats (10^6 cells/rat). The increase in survival time compared with controls is a measure of the number of cells killed in the incubate.
%IST = Percentage increase in survival time as compared with controls.

phamide (Fig. 2a) and the incubate was relatively non-toxic to tumour cells. However, when NADPH and microsomes were present, two radioactive compounds could be extracted with chloroform of which the major one (Fig. 2b, metabolite 1) was clearly distinguishable from cyclophosphamide.

Although, *in vivo*, cyclophosphamide at an intracellular concentration of ~40 μg/ml caused complete regression of the Walker tumour, under *in vitro* conditions toxicity only appeared at a concentration of ~1600 μg/ml. The addition of washed microsomes to the incubation system caused no increase in toxicity nor did the addition of microsomes and NADH. However, in the presence of microsomes and NADPH, there was extensive activation of the agent with significant toxicity towards tumour cells occurring at a concentration of 20 μg/ml.

Figure 3 shows a direct correlation between the concentration of the metabolite and tumour cell toxicity. Cyclophosphamide (60 μg/ml) was incubated with microsomes and NADPH for 45 min,

allowing full activation. At various times after activation, tumour cells at a known concentration were added to aliquots of the incubate, kept at 37° for 1 hr, and bioassayed as before. Concomitantly, aliquots of the microsomal incubate were extracted with chloroform and analysed by TLC.

When tumour cells were added to the incubate immediately after the activation of cyclophosphamide (i.e. at time 0) a large toxic effect (percentage increase in life span) was observed, and a large amount of the metabolite was present (Fig. 3a). However, if the addition of tumour cells was delayed for 2·5 hr, when the amount of metabolite extractable by chloroform had decreased by ~50 per cent (Fig. 3b), the toxicity of the incubation mixture to the tumour cells had correspondingly decreased. Addition of the tumour cells 4 hr after activation, when little metabolite remained (Fig. 3c), resulted in only a small toxicity. After 24 hr the metabolite could not be detected in the incubate (Fig. 3d) which had only a low residual toxicity.

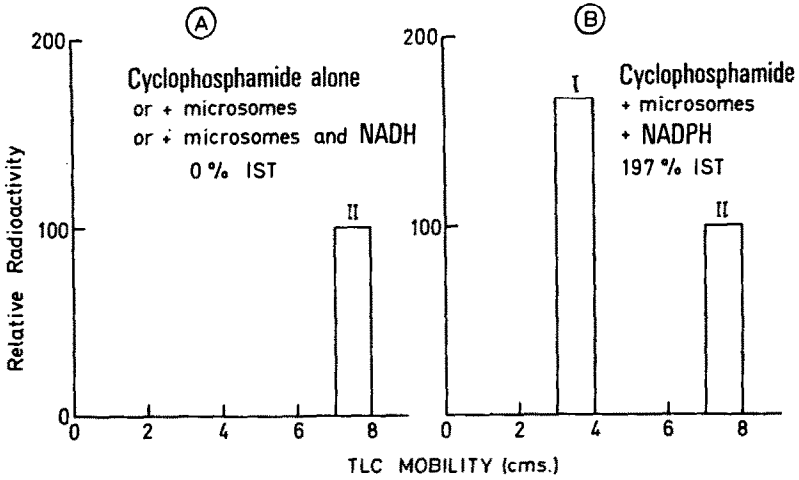

FIG. 2. Cyclophosphamide (60 μg/ml) was incubated in 0·1 M pH 7·4 tris–HCl buffer for 45 min. Microsomes, NADH and an NADPH-generating system were added as required. The incubates were extracted twice with chloroform, and the organic phase was dried ($MgSO_4$) and concentrated to ~0·2 ml. Aliquots (20 μl) were applied to thin-layer plates (silicic acid; Merck: Kieselgel GF_{254}) and developed with butan-1-ol–water (86 : 14). The plates were scanned for radio-activity using a Panax Radiochromatogram Scanner.

I = Metabolite.
II = Cyclophosphamide.

As the amount of metabolite extractable with chloroform decreased, there was a corresponding increase in the concentration of a phosphorus containing ionic substance in the incubation medium. Its electrophoretic mobility (6×10^{-3} cm/V/hr) in buffer of pH 1·85, was consistent with decomposition of the extractable metabolite (mobility 2×10^{-3} cm/V/hr) with the liberation of an ionizable phosphate group.

Preliminary studies involving the isolation of the metabolite and its ionic breakdown product by TLC have indicated that, whereas the former was extremely toxic when incubated with tumour cells, the latter was not.

It is suggested that these findings offer an explanation of the difficulties previously encountered in the isolation of the active metabolite of cyclophosphamide. It has been shown that cyclophosphamide can be activated *in vitro* by microsomal preparations of rat liver provided NADPH is present.[5,7] The results in this paper confirm these findings. However, monitoring of cyclophosphamide metabolism by means of the bioassay system, has now made possible the isolation of an unstable metabolite which is extremely toxic to tumour cells. It therefore has the properties expected of the metabolite responsible for the anit-tumour effects of cyclophosphamide *in vivo*.

Since the metabolite decomposed in a few hours, even in some organic solvents, it is unlikely that it would be insoluble from urine or by any method where a considerable period elapses between activation of cyclophosphamide and isolation of the metabolite.

The structure of the metabolite is under active investigation.

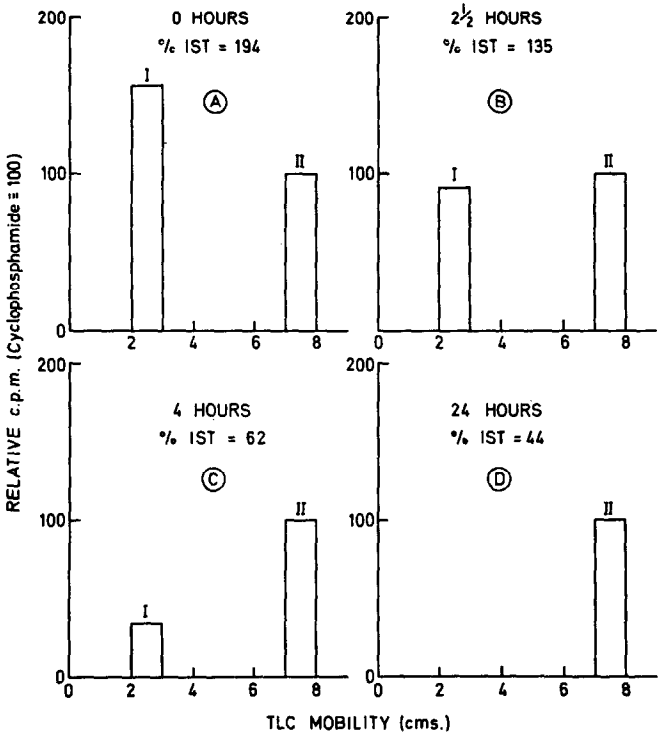

FIG. 3. Cyclophosphamide (60 μg/ml) was incubated with microsomes and an NADPH-generating system in 0·1 M tris–HCl buffer pH 7·4 for 45 min at 37°. At intervals after this time, 5-ml aliquots of the incubation medium were extracted twice with chloroform. The extract was dried ($MgSO_4$) and concentrated to ~0·2 ml. Aliquots (20 μl) were applied to thin-layer plates, chromatographed and scanned as described in Fig. 2.

I = Metabolite.
II = Cyclophosphamide.
%IST = Percentage increase in survival time as compared with controls.

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REFERENCES

1. *Cancer Chemotherapy Rep.* **51**, 315 (6) (1967).
2. N. BROCK, M. ARNOLD and F. BOURSEAUX, *Angew. Chem.* **70**, 539 (1958).
3. H. J. HOHORST, A. ZIEMANN and N. BROCK, *Arzneimittel-Forsch.* **15**, 432 (1965).
4. D. L. HILL, M. C. KIRK and R. F. STRUCK, *J. Am. chem. Soc.* **92**, 3207 (1970).
5. D. L. HILL, *Fedn Proc. Abstracts*, 267 (1971).
6. T. A. CONNORS, A. M. MCLOUGHLIN and P. L. GROVER, *Biochem. Pharmac.* **19**, 1533 (1970).
7. J. L. COHEN and J. Y. JAO, *J. Pharmac. exp. Ther.* **174**, 206 (1970).
8. N. E. SLADEK, *Cancer Res.* **31**, 901 (1971).