Biochemical Pharmacology, Vol. 31, No. 22, pp. 3535–3541, 1982.
Printed in Great Britain.

0006-2952/82/223535-07 $03.00/0

INTERACTION OF CYCLOPHOSPHAMIDE METABOLITES WITH MEMBRANE PROTEINS: AN *IN VITRO* STUDY WITH RABBIT LIVER MICROSOMES AND HUMAN RED BLOOD CELLS

EFFECT OF THIOLS*

DIETER B. WILDENAUER and CHRISTIANA E. OEHLMANN

Institut für Pharmakologie und Toxikologie der Ludwig Maximilian Universität, Medizinische Fakultät, Nussbaumstrasse 26, D-8000 München 2, Federal Republic of Germany

(*Received* 18 *January* 1982; *accepted* 9 *April* 1982)

Abstract—Cyclophosphamide metabolites have been generated *in vitro* by microsomal oxidation of cyclophosphamide and their binding to rabbit liver microsomes and to intact human red blood cells has been investigated. Reactions with proteins of membrane and cytoplasm were detected by SDS polyacrylamide gel electrophoresis. The protein bands were analysed for incorporation of radioactivity. The following results were obtained. (1) Preferential binding of acrolein to microsomes and erythrocytes, with only little binding of metabolites containing the chloroethyl moiety. (2) Reduction of acrolein binding by the thiol compounds glutathione, 2,3-dimercaptopropane-1-sulfonate and mercaptoethane sulfonate. (3) In microsomes: formation of protein polymerisation products and incorporation of radioactivity. (4) In red blood cells: cross-linking of membrane proteins and formation of globin dimerization products in the cytoplasm.

The potent anticancer drug cyclophosphamide (Cp)† requires activation *in vivo* by the microsomal cytochrome P-450 system in order to act as an alkylating agent [1–7, for review see 8,9]. The chloroethyl groups of the activated compound (Fig. 1) are believed to be responsible for the antitumor activity. It has been found, however, that in a nonenzymatic reaction (Fig. 1) acrolein is generated [10]. Several lines of evidence indicate that this metabolite reacts with proteins and causes the toxic side effects of Cp, e.g. haemorrhagic cystitis [11–13]. This has been conclusively demonstrated by Brock *et al.* [12] who identified acrolein as the causative factor of the urotoxic side effects of cyclophosphamide and related compounds. Sulfhydryl groups containing agents like *N*-acetyl-L-cysteine [14–18], 2,3-dimercaptosuccinic acid [19], mercaptoethane sulfonate

* A preliminary report was given at the 22nd Spring Meeting of the German Pharmacological Society at Mainz, 10–13 March 1981 [40].

† Abbreviations used: Cp, cyclophosphamide; 4-OH-Cp, 4-hydroxy-cyclophosphamide; DMPS, 2,3-dimercaptopropane-1-sulfonate; MESNA, mercaptoethane sulfonate; DIMESNA, disulfide of MESNA; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

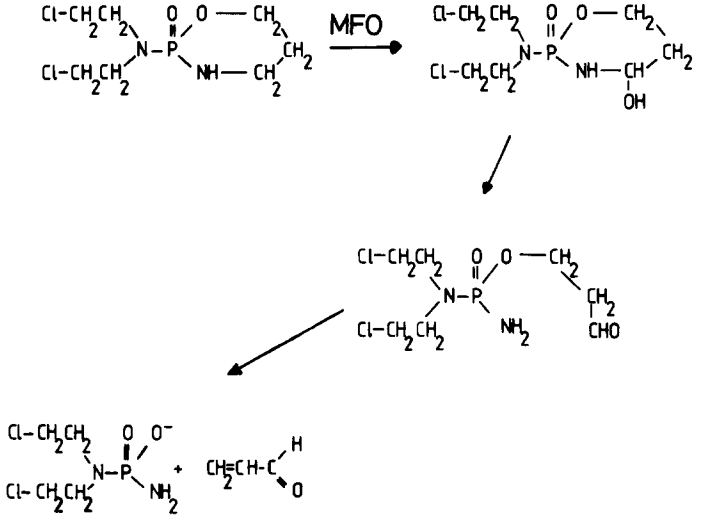

Fig. 1. Metabolism of cyclophosphamide by hepatic microsomal mixed function oxygenase (MFO) as proposed by several authors [31, 37, 38]. A comprehensive summary of the metabolism and its implication on cancerotoxic selectivity has been given by Brock and Hohorst [39].

(MESNA, Uromitexan®, [11, 20]) are able to protect against the action of acrolein *in vitro*, but only MESNA is uroprotective when administered i.v. or orally.

Some work exists on the reaction of cyclophosphamide metabolites with microsomes *in vitro* [18, 21, 22]. Gurtoo *et al.* [21] investigated the binding of [^{3}H-chloroethyl]Cp and [^{14}C-4]-Cp to microsomal proteins and to native calf thymus DNA. These *in vitro* studies show that metabolites containing the chloroethyl groups react preferentially with DNA. Reaction with microsomal proteins, however, appear to be due to the metabolite acrolein. This finding was confirmed in the present investigation and was studied in more detail by analysing the changes in the pattern of microsomal proteins as detected by SDS polyacrylamide gel electrophoresis.

Our previous work has been concerned with the reaction of alkylating agents with membrane proteins employing intact human red blood cells as an *in vitro* model [23, 24]. In the present paper this system was used to study the reactivity of cyclophosphamide metabolites with intact cells *in vitro*. Furthermore, several thiol groups containing compounds such as reduced glutathione, 2,3-dimercaptopropane-1-sulfonate, mercaptoethane sulfonate and its disulfide were tested in this *in vitro* system for their capabilities to reduce the binding of acrolein to proteins.

MATERIALS AND METHODS

Cyclophosphamide (ring-4-^{14}C) was purchased from New England Nuclear, Dreieich, F.R.G. (sp. act. 52.5 mCi/mmole). Cyclophosphamide (^{3}H-labeled in the chloroethyl groups, sp. act. 141 mCi/mmole) and non-labeled Cp were a generous gift from Prof. N. Brock, Astawerke (Bielefeld, F.R.G.). 2,3-Dimercaptopropane-1-sulfonate was obtained from Heyl (Berlin, F.R.G.).

Reduced glutathione was bought from Boehringer (Mannheim, F.R.G.). MESNA and DIMESNA were kindly supplied by Astawerke (Bielefeld, F.R.G.). Material for SDS-polyacrylamide gel electrophoresis was bought from Bio-Rad (München, F.R.G.). All other reagents were analytical grade from E. Merck (Darmstadt, F.R.G.).

Preparation of rabbit liver microsomes [25]. Liver microsomes were prepared from phenobarbital-treated rabbits (50 mg/kg body wt, injected subcutaneously for 1 week) by homogenization of the liver in 0.15 M KCl solution, followed by centrifugation at 9000 g_{max} for 20 min. The supernatant was centrifuged at 100,000 g_{max} for 60 min. The pellet was resuspended in 150 mM phosphate buffer, pH 7.4. The protein content was determined using the biuret procedure [26]. For determination of the cytochrome P-450 content, a sample was reduced with sodium dithionite and treated with CO. The difference spectra were recorded with a Cary 219 spectrophotometer.

Incorporation of cyclophosphamide metabolites into rabbit liver microsomes. Microsomes (1.75 mg protein = 7 nmole cytochrome P-450) were incubated at 37° in 1 ml of 150 mM potassium phosphate buffer pH 7.4 containing 4 mM-[^{14}C]-Cp (sp. act. 0.5 mCi/mmole), 1.5 mM NADP, 12 mM glucose-6-phosphate, 3 mM magnesium chloride, 0.01 mg glucose-6-phosphate dehydrogenase. Aliquots (100 μl) were taken and the protein precipitated by adding 500 μl 0.6 N $HClO_4$ solution and centrifuging at 12,000 g_{max} for 2 min. The pellet was washed twice with 500 μl distilled water each by centrifugation and then dissolved in solubilization buffer as described in the section 'SDS PAGE'.

Incorporation of cyclophosphamide metabolites into human red blood cells. Human blood from healthy donors was centrifuged for 10 min at 2500 g_{max} at 4°. Plasma and buffy coat were removed by suction and the remaining red blood cells were washed three times with phosphate buffered saline, containing 150 mM NaCl and 5 mM phosphate pH 7.4 by centrifugation.

The incubation mixture consisted of microsomes (7 nmole cytochrome P-450/ml), 4 mM ^{14}C-labeled cyclophosphamide (0.125 mCi/mmole) or 4 mM ^{3}H-labeled cyclophosphamide (0.56 mCi/mmole), 1.5 mM NADP, 12 mM glucose-6-phosphate, 3 mM magnesium chloride, 0.01 mg/ml glucose-6-phosphate dehydrogenase in 106 mM potassium phosphate, pH 7.4. Packed red blood cells were added to make a haematocrit of 10%. The thiol compounds were added in a concentration of 4 mM. The samples were incubated at 37° in a water bath and rotated during incubation time. Aliquots (2.5 ml) were taken after different time intervals and the erythrocytes sedimented by centrifugation at 2500 g_{max} for 10 min. The cells were then washed with the incubation buffer and haemolysed in 5 mM phosphate buffer pH 8.0 [27]. The membranes (ghosts) were sedimented by centrifugation at 22,000 g_{max} for 20 min and washed three times with the haemolysis buffer. Radioactivity was determined in the lysate before centrifugation and in the ghost preparation. For this, the samples were decolorized by addition of 0.5 ml H_2O_2 (30%), and 1.5 ml toluene 100, and were counted in a Packard Tricarb Model 3385 using Instagel as scintillation fluid. A sample of ghosts and of haemolysate, after removal of the ghosts, was diluted with solubilization buffer for 'SDS PAGE'.

The supernatant of the incubation mixture was centrifuged for 1 hr at 100,000 g_{max} in order to separate the microsomes. The pellet was washed twice by resuspending it in the incubation buffer and by centrifugation. Radioactivity was determined in the microsomal pellet as described above for lysate and ghosts. A sample was dissolved in solubilization buffer for 'SDS PAGE'. The protein content of lysate, ghosts, and microsomes was determined according to Lowry *et al.* [28].

Incorporation of acrolein into human red blood cells. Washed human erythrocytes (10% haematocrit) were incubated in 106 mM potassium phosphate buffer pH 7.4 with 2.0 mM acrolein at 37°. Aliquots of 2.5 ml were taken after different time intervals, the cells centrifuged, washed and the membranes isolated as described in the preceding section. A sample of this ghost preparation was dissolved in solubilization buffer as described under 'SDS PAGE'.

SDS-polyacrylamide gel electrophoresis. The sample (ghosts, haemolysate, microsomes) was solubilized in a solubilization buffer containing 0.0265 M

Tris, pH 6.8, 2% SDS, 10% glycerol and 5% 2-mercaptoethanol. The solution was kept at 37° for 2 hr and subjected to SDS PAGE on a slab gel. The gels were prepared according to Laemmli [29] using polyacrylamide concentrations of 8.5 and 12%, respectively, for the separating gel and 5% for the stacking gel. The gels were stained with Coomassie blue. For autoradiography the stained gels were dried and exposed for several days to a Kodak medical X-ray film RP/R54.

Another way to determine radioactivity was to slice the gel into 3 mm pieces. Ten ml of 3% protosol in econofluor was added and the samples shaken overnight at 37°. Then liquid scintillation counting was carried out in a Packard Tricarb Model 3385.

RESULTS

Incorporation of metabolites into microsomes and human red blood cells

The reaction of cyclophosphamide metabolites with proteins was studied using either ^{3}H-labeled drug (labeled in the chloroethyl moieties) or ^{14}C-labeled (labeled in the 4-position of the ring). Thus incorporation of ^{3}H-radioactivity must be due to reaction with metabolites containing the tritiated chloroethyl groups. If, however, the amount of ^{14}C-radioactivity exceeds that of the ^{3}H-radioactivity, reaction with metabolites which lack the chloroethyl groups must have occurred.

Only little incorporation or uptake of ^{3}H-activity was found in microsomes (Table 1, Fig. 2A) or

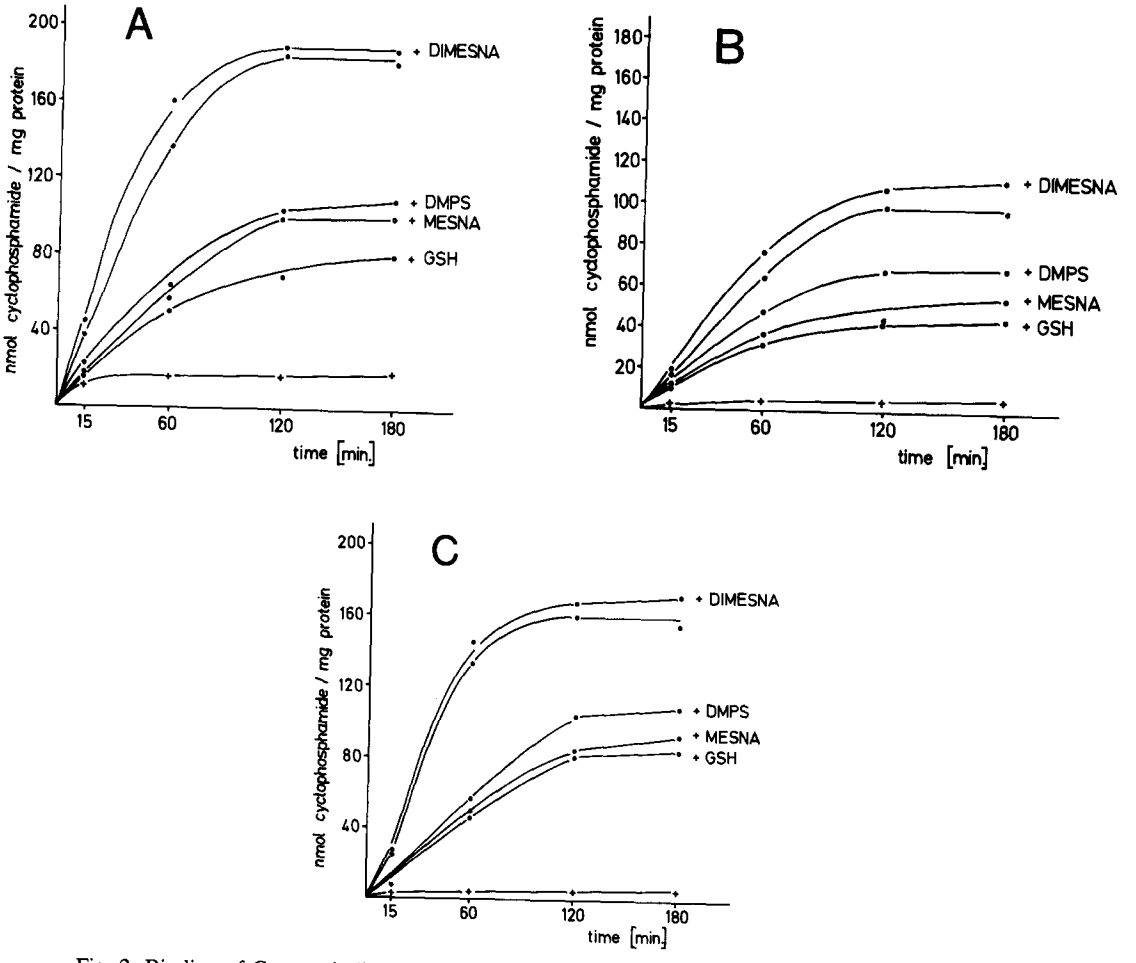

Fig. 2. Binding of Cp metabolites to rabbit liver microsomes (A), human red blood cells (B) and red blood cell membranes (C). The incubation mixture (12.5 ml) contained in 1 ml: 1.75 mg microsomes (7 nmole cytochrome P-450, 4 mM cyclophosphamide [either ^{14}C- (0.125 mCi/mmole) or ^{3}H-(0.56 mCi/mmole) labeled], 1.5 mM NADP, 12 mM glucose-6-phosphate, 0.01 mg glucose-6-phosphate dehydrogenase, 3 mM $MgCl_2$, 106 mM potassium phosphate pH 7.4, washed human red blood cells (10^9 cells), and the thiols in a concentration of 4 mM. The mixture was incubated at 37° for 180 min. After 15, 60, 120 and 180 min aliquots of 2, 5 ml were taken and the red blood cells sedimented by centrifugation. The microsomes were isolated from the supernatant by centrifuging at 100,000 g_{max} for 1 hr. The red blood cells were washed and haemolyzed as described in Materials and Methods. The membranes were isolated from the haemolysate by centrifuging at 27,000 g_{max} for 20 min and washed three times with the haemolysis buffer. Radioactivity was determined in microsomes in the haemolysate before centrifugation and in the red blood cell membrane preparation. The protein content was determined according to Lowry *et al.* [28]. (● - - - ●) binding of ^{14}C-labeled Cp metabolites, addition of thiol compounds as indicated; (+——+) binding of ^{3}H-labeled Cp metabolites.

Table 1. Per cent radioactivity associated with human red blood cells and microsomes after 120 min incubation with 4 mM ^{14}C- or ^{3}H-labeled cyclophosphamide

	[^{14}C]-Cp	[^{14}C]-Cp + 4 mM MESNA	[^{3}H]-Cp
% Radioactivity in red blood cells	80 (33)	39.5	4.4 (1.3)
% Radioactivity in microsomes	7.4 (4.5)	4.2	0.6 (0.3)

The experimental details are given in the legend to Fig. 2.

The data are representative for at least 3 experiments. The values in parentheses were obtained by substitution of the phosphate buffer by 100 mM Tris, pH 7.4, containing 55 mM NaCl.

erythrocytes (Table 1, Fig. 2B, C) suggesting that the metabolites which contain the chloroethyl groups attributed only very little to the reaction of cyclophosphamide metabolites in this system. Substitution of phosphate buffer by Tris buffer did not enhance the incorporation of ^{3}H-activity (Table 1, values in parentheses). The high amount of incorporated ^{14}C-radioactivity (Table 1, Fig. 2A) indicated extensive binding of acrolein which is generated by decomposition of 4-OH-cyclophosphamide (Fig. 1). Uptake of ^{14}C-labeled metabolites was clearly reduced by addition of sulfhydryl-group-containing agents. DMPS, reduced glutathione, and MESNA showed about equally good inactivation of acrolein (Fig. 2A–C). DIMESNA, which has been found in the plasma as the oxidation product of MESNA [30] had, as expected, no effect on the incorporation of ^{14}C-radioactivity into microsomes and red blood cells (Fig. 2A–C). Membranes seem to bind more ^{14}C-labeled metabolites than cytoplasmic components as

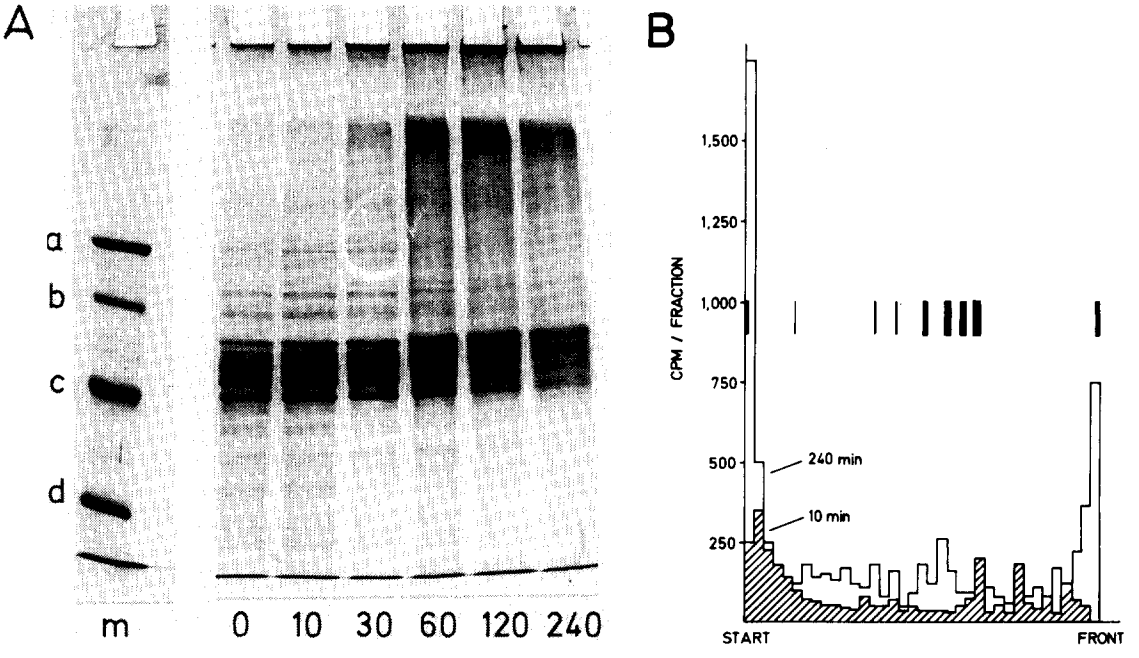

Fig. 3. SDS-polyacrylamide gel electrophoresis (8.5%) of rabbit liver microsomes (A) and distribution of radioactivity in the gel (B) after incubation with 4 mM cyclophosphamide. Microsomes (1.75 mg protein = 7 nmole cytochrome P-450) were incubated for 240 min at 37° in 1 ml of 150 mM potassium phosphate buffer pH 7.4, containing 4 mM ^{14}C-labeled cyclophosphamide (sp. act. 0.5 mCi/mmole), 1.5 mM NADP, 12 mM glucose-6-phosphate, 3 mM $MgCl_2$, 0.01 mg glucose-6-phosphate dehydrogenase. Aliquots of 100 μl were taken after 0, 10, 30, 60, 120 and 240 min. The protein was precipitated with potassium perchlorate and solubilized as described in Materials and Methods. Aliquots (protein content about 20 μg) were applied to a 8.5% acrylamide containing slab gel (prepared according to Laemmli [29]). (A) Gel stained with Coomassie blue. The numbers on the bottom indicate different incubation times. m, marker proteins (a = phosphorylase, mol. wt 92,500; b = bovine serum albumin, mol. wt 66,200; c = ovalbumin, mol. wt 45,000; d = carbonic anhydrase, mol. wt 31,000). (B) Distribution of ^{14}C-activity in the gel. The gel was sliced vertically into 10 mm pieces and horizontally into 3 mm pieces and solubilized as described in Materials and Methods. Filled columns, after 10 min incubation as described above; open columns, after 240 min incubation. The position of bands staining with Coomassie blue is schematically drawn on the upper part of the graph.

can be seen from a comparison of the data for microsomes and ghosts with the values for the whole red blood cells (Fig. 2A–C).

SDS PAGE

A gel loaded with solubilized microsomes which had been pretreated with 4 mM cyclophosphamide and incubated for different time intervals, showed a decrease of the cytochrome P-450-bands and the appearance of polymerisation products on top of the gel (Fig. 3A). When ^{14}C-labeled cyclophosphamide was used, radioactivity could be detected in the gel (Fig. 3B). No radioactivity could be found when ^{3}H-labeled cyclophosphamide was used in the same system (not shown). This suggests that the polymerisation products were generated by the action of acrolein.

Figure 4 shows the result of the reaction of Cp with membrane proteins in intact human red blood cells. When the membrane proteins were analysed by SDS PAGE and stained with Coomassie blue, reaction with the spectrin units 1 and 2 was apparent (Fig. 4A). The bands became more diffuse and polymerisation products appeared on top of the gel as in the case of microsomes. Autoradiography (Fig. 4B) revealed the incorporation of ^{14}C-radioactivity in these regions. No radioactivity was found using ^{3}H-labeled Cp (not shown). Besides labeling of spectrin, some radioactivity was incorporated into other protein bands (Fig. 4B). A higher amount of ^{14}C-activity was found in the protein which migrated with the front and consisted mainly of globin. There was also some radioactivity ahead of the tracking dye which could be due to phospholipid labeling (Fig. 4B).

A very similar pattern has been found earlier for the reaction of red blood cells with the mustard gas derivative HN3 [23]. However, since no tritium activity could be detected using Cp, labeled in the chloroethyl groups, it was concluded that the modification by Cp is due to acrolein. When washed human red cells were incubated with 2 mM acrolein for different time intervals, a similar pattern was obtained (Fig. 5).

Reaction with components of the cytoplasm could be detected in the haemolysate using SDS PAGE (Fig. 6). A diffuse band appeared in a position above the band for carbonic anhydrase (Fig. 6). This pro-

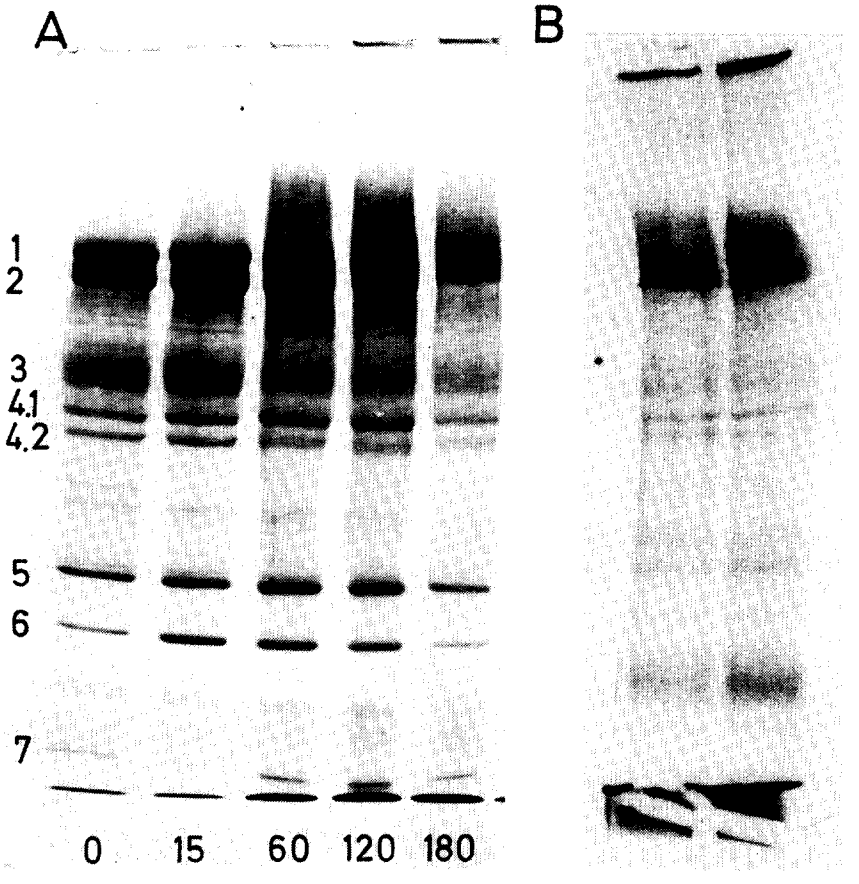

Fig. 4. SDS-polyacrylamide gel electrophoresis (7.5%) of red blood cell membranes. A part of the membrane preparation described in the legend to Fig. 2 was dissolved in solubilization buffer and subjected to SDS PAGE on a Laemmli gel as described in Materials and Methods. (A) Gel stained with Coomassie blue; the numbers on the bottom indicate different incubation times. The nomenclature of the bands has been adapted from Steck [32]. (B) Autoradiography of a dried gel. The membranes were prepared from an incubation mixture as described in Fig. 2, except that [^{14}C]-Cp was used with a sp. act. of 1.1 mCi/mmole.

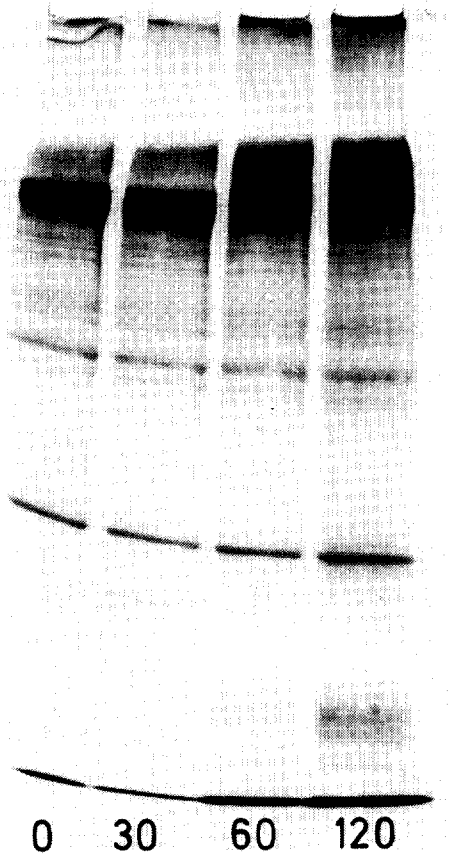

Fig. 5. Reaction of 2 mM acrolein with erythrocyte membrane detected by SDS PAGE. Washed human red blood cells were incubated with 2 mM acrolein in 106 mM potassium phosphate buffer pH 7.4 at 37°. After 0, 30, 60 and 120 min 2 ml aliquots were taken, the erythrocytes sedimented and membranes prepared as described in Materials and Methods. The preparation was dissolved in solubilization buffer and aliquots containing about 20 μg protein were applied to a slab gel containing 7.5% acrylamide. The numbers on the bottom indicate different incubation times.

tein has been identified earlier as a globin dimer, generated by intramolecular cross-linking of hemoglobin [23].

DISCUSSION

The finding that reaction of the Cp-metabolite acrolein with proteins is responsible for the Cp caused haemorrhagic cystitis [12] lends importance to a study of this reaction at the molecular level. In the present report we used human red blood cells as a model for interaction with proteins.

Reaction of ^{14}C-labeled Cp-metabolites with membrane and cytoplasmic proteins in intact cells was demonstrated *in vitro*. In contrast, when Cp with tritiated chloroethyl groups was used, only little ^{3}H-incorporation could be detected. To avoid reactions of the nucleophile phosphate with the chloroethyl groups, the incubation was carried out in Tris buffer (Table 1, values in parentheses). The difference in incorporation of ^{14}C- and ^{3}H-activity remains similar, although lower values were obtained in Tris buffer. From these results we concluded that mainly acrolein, which is formed nonenzymatically from 4-OH-Cp (Fig. 1), was bound to the proteins. This indirect appraoch was taken since quite a number of reaction partners can be involved in the binding of acrolein and, therefore, chemical analysis of the reaction products would have been a difficult task.

The amount of ^{14}C-radioactivity which was found associated with the red blood cells (Table 1) after 2 hr *in vitro* incubation was surprisingly high, especially since the incubation mixture contained Mg^{2+} which is supposed to prolong the $t_{1/2}$ of 4-OH-Cp [31]. Binding and uptake of acrolein into intact erythrocytes seems to be favoured when compared to the Cp-metabolites which contain the mustard moiety.

It is unlikely that effects of Cp on erythrocytes play a role in the cytotoxic action or in generation of toxic side effects. Nevertheless, red blood cells are a suitable model to study chemical–biological interactions, because the molecular composition of the membrane is well defined and the proteins can be analysed by SDS PAGE [32, 33]. Moreover, since the Cp-metabolites were transported (after biotransformation in the liver) by the bloodstream, erythrocytes as well as serum proteins might serve as scavengers for the generated acrolein and prevent the transport to more sensitive targets. Since the

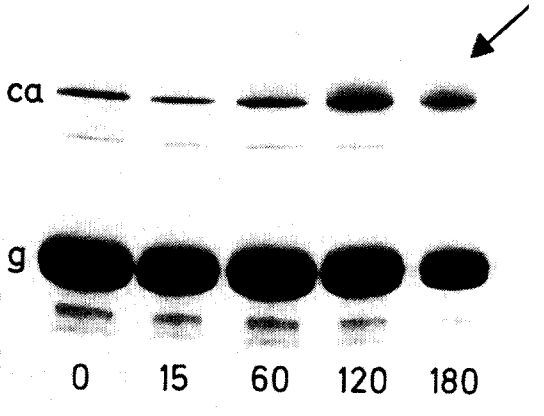

Fig. 6. SDS-polyacrylamide gel electrophoresis (12%) of the haemolysate. A part of the haemolysate from the experiment described in the legend of Fig. 2 was diluted with solubilization buffer and subjected to SDS PAGE. The numbers on the bottom indicate different incubation times. g, globin; ca, carbonic anhydrase; the arrow indicates the appearance of a globin dimerization products.

urine is virtually protein free, acrolein, which is liberated after glomerular filtration of the hydroxylated metabolites, is not inactivated and attacks exclusively the renal and vesical epithelia [12].

The most pronounced effect was the reaction with spectrin (Fig. 4, bands 1 and 2), a protein of the cytoskeleton. The cytoskeleton appears to be important for maintenance of the cell shape [24, 33–35] and seems to be involved in certain transmembrane effects like control of receptor binding [35, 36].

Changes in the structural organisation of the cytoskeleton might affect certain functions or even the viability of the cell. Cytoskeleton like structures have been found and characterized in many other cells.

Formation of polymerisation products was also detected when the microsomal proteins were analysed by SDS PAGE (Fig. 3). There seems to be no specificity in the reaction. Moreover, since binding of ^{3}H-activity was only very small (Fig. 2A), the first metabolite of Cp, 4-OH-Cp, did not seem to bind covalently in an appreciable amount to the metabolizing enzyme.

Thiols have been used in the therapy with cyclophosphamide in order to reduce the urotoxic side effects caused by acrolein. The mode of acrolein inactivation by thiols has been studied by Brock *et al.* [12] for MESNA and found to occur by formation either of a thiol-acrolein adduct or of a thiol-4-Cp condensation product which reduces the speed of acrolein formation. In the *in vitro* system here described, it was possible to demonstrate a good reduction of the reactivity of the Cp metabolite acrolein by the thiol compounds glutathione, DMPS and MESNA. DIMESNA which is formed *in vivo* by oxidation of MESNA was, as expected, inactive. It has been demonstrated very elegantly by Ormstad *et al.* [30] that DIMESNA is transported to the kidney and specifically taken up by this organ. In the kidney, DIMESNA is reduced back to MESNA which is the active thiol compound. Thus, MESNA seems to be the superior agent for the regional detoxification of the urogenital tract without diminishing the cytostatic potency of Cp.

Acknowledgements—This work has been supported by a grant from the Curt-Bohnewand-Fonds. The authors wish to express their gratitude to Prof. N. Brock for a gift of tritiated cyclophosphamide and to Dr. J. Pohl for valuable comments. We are very grateful to Susan Metzger for critical reading of the manuscript.

REFERENCES

1. N. Brock, R. Gross, H.-J. Hohorst, H. O. Klein and B. Schneider, *Cancer* **27**, 1512 (1971).
2. N. Brock and H.-J. Hohorst, *Cancer* **20**, 900 (1967).
3. J. L. Cohen and J. Y. Jao, *J. Pharmac. exp. Ther.* **174**, 206 (1970).
4. N. Brock and H.-J. Hohorst, *Arzneimittel Forsch.* **13**, 1021 (1963).
5. G. E. Foley, O. M. Friedman and B. P. Drolet, *Cancer Res.* **21**, 57 (1961).
6. R. F. Struck, M. C. Kirk, L. B. Mellet, S. ElDareer and D. L. Hill, *Molec. Pharmac.* **7**, 519 (1971).
7. T. A. Connors, A. B. Forster, A. M. Gilsenan, M. Jarman and M. J. Tisdale, *Biochem. Pharmac.* **21**, 1373 (1972).
8. D. L. Hill, *A Review of Cyclophosphamide.* Charles C. Thomas, Springfield (1975).
9. O. M. Friedman, A. Myles and M. Colvin, in *Advances in Cancer Chemotherapy* (Ed. A. Rosowsky), p. 143. Marcel Dekker, New York (1979).
10. R. A. Alarcon and J. Meienhofer, *Nature, Lond.* **233**, 250 (1971).
11. N. Brock, J. Stekar, J. Pohl and W. Scheef, *Naturwissenschaften* **66**, 60 (1979).
12. N. Brock, J. Stekar, J. Pohl, U. Niemeyer and G. Scheffler, *Arzneimittel Forsch.* **29**, 659 (1979).
13. P. J. Cox, *Biochem. Pharmac.* **28**, 2045 (1979).
14. E. Harris, L. Levy and J. Levy, *Proc. west. Pharmac. Soc.* **18**, 354 (1975).
15. A. Primack, *J. natn. Cancer Inst.* **47**, 223 (1971).
16. J. A. Botta, Jr., L. W. Nelson and J. H. Weikel, Jr., *J. natn. Cancer Inst.* **51**, 1051 (1973).
17. L. Levy and R. Harris, *Biochem. Pharmac.* **26**, 1015 (1977).
18. M. J. Berrigan, H. L. Gurtoo, S. D. Sharma, R. F. Struck and A. J. Marinello, *Biochem. biophys. Res. Commun.* **93**, 797 (1980).
19. P. J. Cox and G. Abel, *Biochem. Pharmac.* **28**, 3499 (1979).
20. W. Scheef, H. O. Klein, N. Brock, H. Burkart, U. Günther, H. Haefer-Janker, D. Mitrenga, J. Schnitker and R. Voigtman, *Cancer Treat. Rep.* **63**, 501 (1978).
21. H. L. Gurtoo, R. Dahms, J. Hipkens and J. B. Vaught, *Life Sci.* **22**, 45 (1978).
22. A. J. Marinello, H. L. Gurtoo, R. F. Struck and B. Paul, *Biochem. biophys. Res. Commun.* **83**, 1347 (1978).
23. D. Wildenauer and N. Weger, *Biochem. Pharmac.* **28**, 2761 (1971).
24. D. B. Wildenauer, H. Reuther and J. Remien, *Biochim. biophys. Acta* **603**, 101 (1980).
25. R. von Jagow, H. Kampffmeyer and M. Kiese, *Naunyn-Schmiedebergs Archs exp. Pharmac.* **251**, 73 (1965).
26. A. G. Gornall, C. J. Bardawill and M. M. David, *J. biol. Chem.* **177** (1949).
27. T. L. Steck and J. A. Kant, *Meth. Enzym.* **31A**, 172 (1974).
28. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
29. U. K. Laemmli, *Nature, Lond.* **227**, 680 (1970).
30. K. Ormstad, S. Orrenius and N. Brook, 8th International Congress of Pharmacology, 19–24 July (1981). Abstracts, 271.
31. G. Völker, U. Dräger, G. Peter and H.-J. Hohorst, *Arzneimittel Forsch.* **24**, 1172 (1974).
32. T. L. Steck, *J. Cell Biol.* **62**, 1 (1974).
33. v. T. Marchesi and H. Furthmayr, *A. Rev. Biochem.* **45**, 667 (1976).
34. M. P. Sheetz and S. J. Singer, *J. Cell Biol.* **73**, 638 (1977).
35. G. J. Nicolson, *Biochim. biophys. Acta* **457**, 57 (1976).
36. T. H. Ji and G. L. Nicholson, *Proc. natn. acad. Sci. U.S.A.* **71**, 2212 (1974).
37. M. Jarman, *Biochem. Pharmac.* **23**, 115 (1974).
38. D. L. Hill, W. R. Larster and R. F. Struck, *Cancer Res.* **32**, 658 (1972).
39. N. Brock and H. J. Hohorst, *Z. Krebsforsch.* **88**, 185 (1977).
40. D. B. Wildenauer and Ch. E. Oehlmann, *Naunyn-Schmiedeberg's Archs Pharmac.* **316**, Suppl. 29 (1981).