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REACTIONS OF THE TRIFUNCTIONAL NITROGEN MUSTARD TRIS(2-CHLOROETHYL)-AMINE (HN3) WITH HUMAN ERYTHROCYTE MEMBRANES *IN VITRO**

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Abstract—Covalent labeling of membrane proteins and cytoplasmic components in washed human erythrocytes by the alkylating antitumor agent HN3 has been studied *in vitro*. Using ^{14}C-labeled HN3 and SDS-polyacrylamide-gel-electrophoresis for separation of the reaction products the following results have been obtained: (1) The membrane protein spectrin (bands 1,2) disappeared from the SDS-polyacrylamide-gel-electrophoresis pattern. Formation of proteins with higher molecular weight suggested cross-linking of spectrin to itself and to other proteins from membrane and cytoplasm. (2) The carbohydrate containing protein glycophorin (PASI) and the protein in band 7 were labeled by the compound. Other membrane proteins reacted to a lower extent. (3) The alkylating agent penetrated the membrane and reacted with hemoglobin in the cytoplasm. The formation of globin dimers was detected.

Reactions of alkylating agents with DNA are considered to be the mechanism for their mutagenic, carcinogenic and cytotoxic action. This is well established in the case of mutagenicity and very likely applies to the case of carcinogenicity. However, it is not always possible to draw a correlation between cytotoxicity and alkylation of DNA. In some instances, alkylation or damage of DNA was not detected in cells treated with cytotoxic concentrations of alkylating agents [2–5]. Therefore, reactions with other cell constituents may play a role in the cytotoxic effect of these compounds.

The reaction of alkylating agents with proteins, enzymes and metabolites has been studied by several groups. Their work has been extensively reviewed by Ross [6], Ochoa and Hirschberg [7] and more recently by Connors [2]. The effect of HN3† on purified hemoglobin was studied in our laboratory by Albrecht *et al.* [8].

Although some work exists on the interaction of alkylating antitumor agents with cell membranes, little is known about their reaction on the molecular level. Studies on tumors which are sensitive to alkylating agents and on tumors which are resistant revealed altered permeability through cell membranes, one of the mechanisms by which cells acquire resistance to alkylating agents [9–11]. Grunike *et al.* detected a selected inhibition of thymidine transport by the alkylating antitumor agent triethyleneiminobenzochinone [12]. The uptake of *N*-methyl-bis(2-chloroethyl) amine into L 5178 Y lymphoma cells was found to be mediated by the choline transport system [13, 14]. Furthermore, Linford and coworkers [15] showed that chlorambucil interacts with cell surfaces of human erythrocytes causing hemolysis.

The aim of our work is to explore reactions of alkylating agents with membrane components on the molecular level. This paper describes the effects of the trifunctional alkylating agent HN3 on intact human erythrocytes *in vitro*. The erythrocyte membrane was used as a model, since its molecular composition is well defined and can be analyzed by SDS-polyacrylamide-gel-electrophoresis (SDS-PAGE) [16, 17].

Additional aspects of this study are to investigate the usefulness of HN3 as a probe for chemical labeling and cross-linking. Reactions of chemical cross-linking agents with components of intact human erythrocytes have been reported earlier [18–21].

MATERIALS AND METHODS

Materials. [U-^{14}C]Tris-(2-chloroethyl)amine hydrochloride (sp. act. 5 mCi/mmole) was synthesized by Behringwerke AG, Radiochemical Laboratory, Frankfurt/M, GFR. Sodium dodecylsulfate, acrylamide and bisacrylamide were purchased from Bio-Rad. Instagel and Soluene were obtained from Packard. All other chemicals used were reagent grade.

Red blood cells. Human blood was obtained from healthy donors and immediately centrifuged for 10 min at 2500 g_{max} at 4°. Plasma and buffy coat were removed by suction. The remaining red blood cells were washed 3 times with phosphate buffered saline (PBS) containing 150 mM-NaCl, 5 mM-phosphate pH 8.0 and then with 100 mM-Tris buffer pH 7.0 containing 0.37% NaCl by centrifugation (10 min at 2500 g_{max}).

Treatment of erythrocytes with HN3. HN3 was dissolved in 100 mM-Tris buffer pH 7.0 containing 0.37% NaCl. Packed red cells were added to make up a hematocrit of 10%. During incubation at 37° with

* A preliminary report has been given at the 7th International Congress of Pharmacology in Paris, 16–21 July, 1978 [1].

† Abbreviations—HN3 = tris(2-chloroethyl)-amine HCl, Tris = 2-amino-2-hydroxymethylpropane-1:3-diol, PBS = phosphate buffered saline, PAGE = polyacrylamide gel electrophoresis, SDS = sodium dodecyl sulfate, PAS = periodic acid staining, NBP = 4-(4-nitrobenzyl) pyridine, TEMED = N,N,N′,N′,tetramethylethylenediamine, PPO = 2,5-diphenyloxazole, POPOP = 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene.

gentle shaking, aliquots of 5 ml were taken, and the reaction stopped by washing the erythrocytes with ice-cold PBS buffer by centrifugation at 4° (5 min at 5000 g_{max}). For determination of uptake of hydrolysed HN3 by red blood cells HN3 was preincubated in 100 mM-Tris buffer pH 7.0 containing 0.37% NaCl for 2 hr at 37°. Packed red cells were added and the incubation performed as in the case of unhydrolysed HN3.

Hemolysis was determined by diluting 200 μl aliquots of the supernatant with 10 ml distilled water and reading the absorbance at 418 nm against water. For 100% hemolysis the incubation mixture was replaced by water.

Preparation of ghosts. Ghosts were prepared by hypotonic lysis in 5 mM-sodium phosphate buffer pH 8.0 according to Steck and Kant [22]. The lysate was centrifuged at 22,000 g_{max} for 15 min and the pellet was washed 4 times with 5 mM-phosphate buffer pH 8.0. The resulting ghost preparation was immediately used for electrophoresis experiments. Protein content was determined according to Lowry *et al.* [23]. Aliquots were taken for determination of radioactivity and counted in a Packard Tricarb Model 3385 using a dioxane based scintillation fluid (Bray's solution). The radioactivity of the hemolysate was determined in Instagel after decoloration in 1.5 ml Soluene 100–isopropanol mixture (1:1) and 0.5 ml hydrogen peroxide (30%) for half an hour.

SDS-polyacrylamide-gel-electrophoresis. Samples were solubilized in a buffer containing 0.0625 M-Tris pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, 5% 2-mercaptoethanol and boiled for 2 min. The preparation was stored at −20°.

Slab gels were prepared essentially according to Laemmli [24]. The separating gel was polymerized using the following concentrations: 5% (7.5%, 15%) polyacrylamide taken from a stock solution containing 0.8% *N,N'*-bis-methyleneacrylamide, 0.375 M-Tris–HCl pH 8.8, 0.1% SDS, 0.025% tetramethylethylene diamine and 0.025% ammonium persulfate. The stacking gel contained 3% polyacrylamide, 0.125 M-Tris–HCl pH 6.8, 0.1% SDS, 0.025% Temed and 0.025% ammonium persulfate.

The electrophoresis was performed in an apparatus similar to Studier's [25]. The electrophoresis buffer contained 0.025 M-Tris, 0.192 M-glycine pH 8.3 and 0.1% SDS. The gels were run at 35 mA/gel for 3 hr at 0°. Fixation of the gels was done with 25% isopropanol, 10% acetic acid, staining with a solution of 0.01% Coomassie Blue in 25% methanol, 10% acetic acid and destaining by several changes of an aqueous solution of 5% methanol, 7% acetic acid. Carbohydrate containing proteins were stained using a modified PAS technique described by Zacharias *et al.* [26].

For drying the gels were placed onto filterpaper in a Buchner funnel and covered by a thin rubber sheet. A vacuum was applied and the funnel was heated using a fan.

For autoradiography of the dried gels Kodak Medical X-ray film RP/R54 was used.

For determination of radioactivity the gels were sliced vertically in 10 mm and horizontally in 3 mm

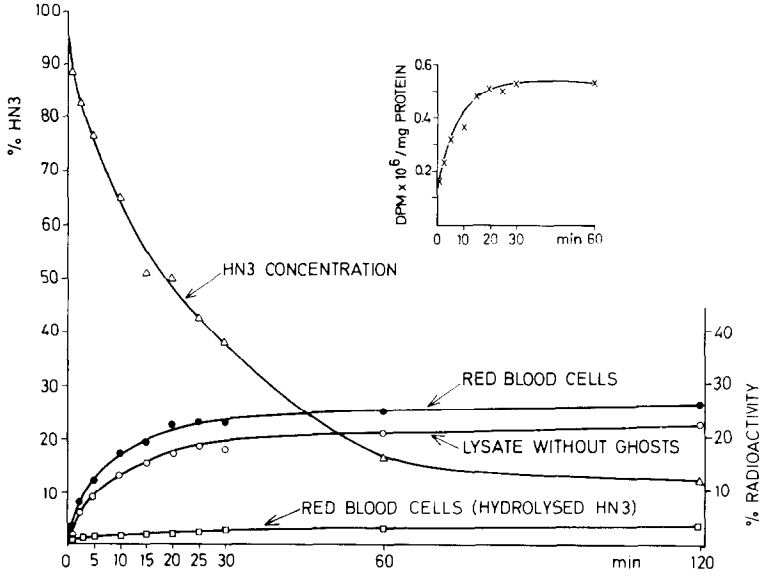

Fig. 1. Rate of uptake of ^{14}C-labeled HN3 and its hydrolysis products by human erythrocytes. Washed human erythrocytes (hematocrit 10%) were incubated in 100 mM Tris buffer pH 7.0, containing 0.37% NaCl at 37° with 1 mM-HN3 or with the hydrolysed compound prepared by preincubation for 2 hr at 37° in the same buffer. 5 ml aliquots were taken and after washing with PBS buffer ghosts were prepared by hypotonic lysis as described in Materials and Methods section. The radioactivity in the lysate was determined before and after removal of the ghosts. ●—● = Per cent radioactivity taken up by red blood cells incubated with HN3; ○—○ = per cent radioactivity remaining in the lysate after removing the ghosts; △—△ = rate of reaction of HN3 determined by the NBP reaction as described in Materials and Methods section; □—□ = per cent radioactivity taken up by red blood cells incubated with hydrolysed HN3. Incorporation of radioactivity into the ghosts is shown in the insert.

Table 1. Uptake and distribution of radioactivity in red cells after 60 min incubation with 1 mM ^{14}C-labeled HN3

	Red cells*	Hemolysate without ghosts†	Ghosts‡	Hemoglobin
Per cent radioactivity	23	17.6	5.4	13
Sp.act. (nmoles/mg protein)	—	—	19.3	5.3

Washed human erythrocytes were incubated with 1 mM ^{14}C-labeled HN3 for 60 min at 37°. Radioactivity was determined by taking aliquots after hypotonic lysis of the washed incubated erythrocytes before (*) and after (†) removal of the ghosts by centrifugation. The incorporation of HN3 into ghosts (‡) was calculated from the differences (* − †). Specific activity was determined in ghosts washed with hypotonic buffer as described in Materials and Methods. Hemoglobin was separated by Sephadex G 200 gel-filtration (Fig.6B, fractions 37–47) from the residual cytoplasmic components. Hemoglobin content was estimated after reaction with CO by measuring the absorbance at 569 nm. Ghost-protein was determined by Lowry's procedure [23].

pieces and counted in toluene containing 10% Soluene 350, PPO and POPOP (0.32 g and 0.08 g, respectively, 100 ml toluene) after being shaken overnight in this mixture.

Gel chromatography. The hemolysate obtained by lysis of erythrocytes incubated with HN3 was applied to a column (100 cm × 2 cm) of Shephadex G 200. The column was eluted with 5 mM-phosphate buffer pH 8.0.

Hydrolysis of HN3. The rate of hydrolysis of the alkylating agent in the incubation mixture was determined according to Workman *et al.* [27]. 0.5 ml of the supernatant derived from centrifugation of HN3-incubated erythrocytes was incubated with 1 ml NBP reagent (2 g 4-(4-nitrobenzyl)-pyridine dissolved in 100 ml 90% ethylene glycol, 10% 0.5 mM-acetate buffer pH 4.6) for 20 min at 90°. After cooling, 3 ml of a 1:1 mixture of triethylamine–acetone was added and the absorbance at 570 nm determined in a Zeiss spectral photometer.

RESULTS

Uptake of HN3 *by erythrocytes.* The incorporation of HN3 into washed human erythrocytes was studied by incubating the erythrocytes at a concentration of 10^{12}/liter with a concentration of 1 mM-^{14}C-labeled HN3. Uptake of radioactivity was rapid during the initial 15 min of incubation at 37° (Fig. 1). Then the uptake slowed down and stopped due to hydrolysis of the reagent and reaction of the reagent with buffer components. After 2 hr incubation with 1 mM-HN3 at 37°, 23 per cent of the radioactivity was found in the intact red cells. The distribution of HN3 between membranes and cytoplasm was calculated from the difference of radioactivity in the lysate before and after removing the membranes. 23.5 Per cent of the radioactivity taken up by the cells was incorporated into the membranes. The 76.5 per cent remaining in the hemolysate after centrifugation represented the amount of HN3 or its hydrolysis products in the cytoplasm. Uptake of hydrolysis products of HN3 (Fig. 1) was studied after preincubation of ^{14}C-labeled HN3 in the incubation mixture for 2 hr at 37°. 3.7 Per cent of the radioactivity was taken up by the red blood cells. The specific activity of the ghosts after 60 min incubation as described in the experimental section was 19.3 nmole HN3 per mg protein (Table 1). Reaction of HN3 with phospholipids was not detected by thin-layer chromatography [28] and autoradiography (not shown) of the lipids extracted from ghost membranes by the Bligh and Dyer procedure [29]. The rate of hemolysis was determined and found to be less than 1 per cent. It did not exceed the rate obtained in a control experiment with erythrocytes incubated in the absence of alkylating agent for two hr at 37°.

Reaction of HN3 *with membrane proteins.* The most striking result of the reaction was the change in spectrin as monitored by SDS-PAGE of the erythrocyte membranes. Electrophoresis of solubilized ghosts prepared from HN3 treated erythrocytes on a 5% polyacrylamide gel in SDS (Fig. 2A) showed the disappearance of the spectrin bands (band 1 and 2 in Fig. 2A) and the appearance of bands containing proteins with higher molecular weight. The position of the new protein bands suggests not only cross-linking between spectrin bands 1 and 2, but also between spectrin and polypeptides with lower molecular weight. The bands appearing just above the spectrin bands with an increased molecular weight of about 10,000–20,000 are probably the products of cross-linking between hemoglobin and spectrin. This interpretation is in agreement with results obtained by treatment of hemoglobin-free ghosts with HN3. SDS-polyacrylamide gels of this preparation did not show these bands (results not shown). Longer incubation of erythrocytes with HN3 produces polymers which did not enter the gel.

The autoradiograph (Fig. 2B) of the 5% polyacrylamide gel shows that incorporation of radioactivity into the spectrin components occurred within 1 min of incubation with ^{14}C-labeled HN3 and was followed by the production of radioactively labeled compounds of higher molecular weight. This result indicates that the cross-links between protein molecules were generated by HN3.

The gel pattern of other membrane proteins as detected by Coomassie Blue or carbohydrate staining did not show a drastic change comparable to that of spectrin after reaction with HN3 (Fig. 3A,C). The bottom band which contains globin became more intense, since ghosts prepared from HN3-incubated erythrocytes retained more hemoglobin than ghosts from untreated red

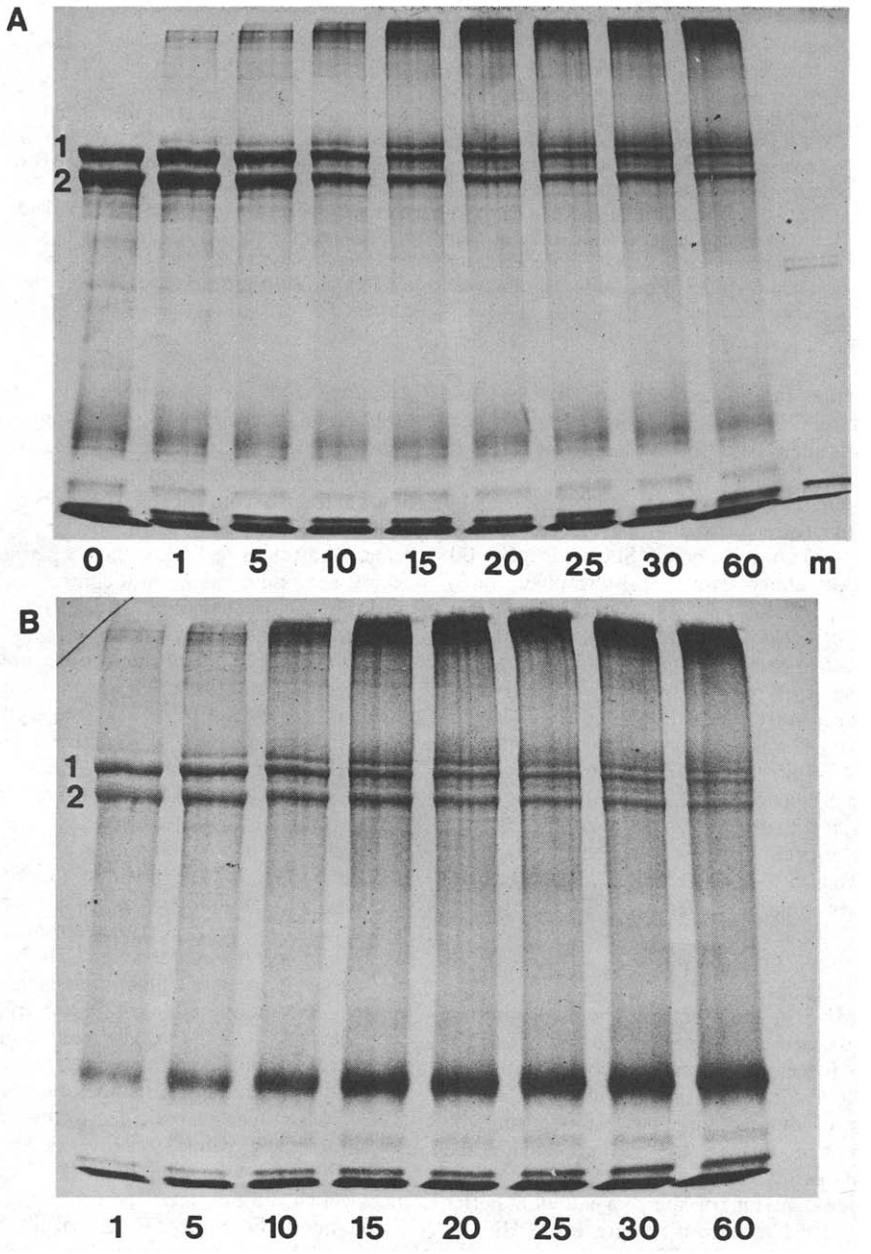

Fig. 2. 5% SDS-polyacrylamide-gel-electrophoresis of erythrocyte membrane proteins after reaction with HN3. Erythrocytes were incubated with 1 mM ^{14}C-labeled HN3 and ghosts were prepared and solubilized as described in Materials and Methods section. 20 μg of protein determined according to Lowry [23] was applied to each sample well. 1,2 = spectrin (the nomenclature has been adapted from Steck [17]). The numbers on the bottom indicate different incubation times in minutes. m = marker protein (RNA polymerase, β,β'-subunits, average m.wt = 160,000). A: gel stained with Coomassie Blue; B: autoradiograph of the dried gel.

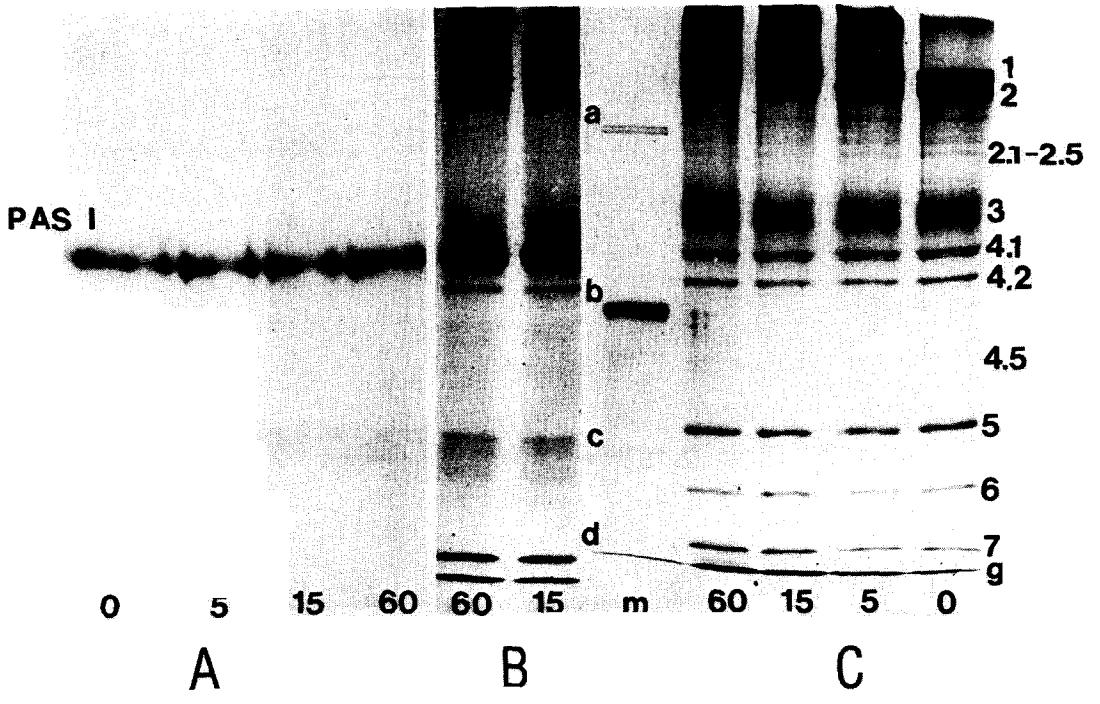

Fig. 3. 7.5% SDS-polyacrylamide-gel-electrophoresis. The conditions for incubation, preparation of membranes and solubilization were the same as in Fig. 2, except for A, where 60 μg of protein was applied to the gel. A: gel stained by the PAS procedure; B: autoradiograph; C: gel stained with Coomassie Blue. The numbers on the bottom indicate different incubation times in minutes. m = marker proteins (a = β,β'-subunits of RNA polymerase, average m.wt = 160,000; b = bovine albumin, m.wt = 68,000; c = α-subunit of RNA polymerase, m.wt = 39,000; d = trypsin inhibitor, m.wt = 21,500). The nomenclature of the bands has been adapted from Steck [17]. 1,2 = spectrin, 5 = actin, 6 = subunit of PGD, g = globin, PAS I = glycophorin.

cells. Some minor bands (2.1–2.5, 4.5) that disappeared were presumably converted by cross-linking to the products with higher molecular weight which appeared at the top of the gel. Several other membrane proteins were labeled by the alkylating agent as shown in the autoradiograph of the dried gel (Fig. 3B).

A substantial amount of radioactivity was found in a band which corresponds to the carbohydrate-containing protein PAS I (glycophorin, Fig. 3A) and in band 7. The proteins in bands 4.2 and 5 (actin) are labeled to a lesser extent (Fig. 4), although the amount of these proteins in the membrane is about the same (17) as that of PAS I and band 7.

Reaction of HN3 *with components of the cytoplasm.* During incubation of intact human erythrocytes with HN3 a polypeptide with a molecular weight of 32,000 was formed in the cytoplasm (Fig. 5A). A protein with the same apparent molecular weight, when analyzed by SDS-PAGE (Fig. 5A) was obtained by alkylation of purified hemoglobin with HN3 as described by Albrecht *et al.* [8]. This suggests that the protein appearing during alkylation is generated by cross-linking between two globin chains. The autoradiograph (Fig. 5B) of the gel shows incorporation of radioactivity into globin and into the globin dimer.

To separate the products of the reaction between HN3 and the cytoplasmic components red cells were incubated with 1 mM HN3 for 60 min and a portion of

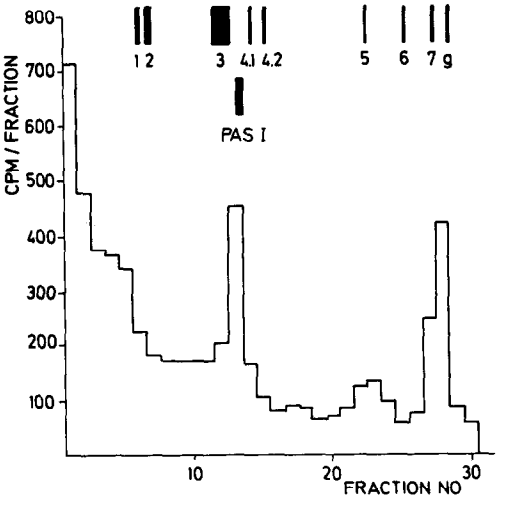

Fig. 4. Distribution of radioactivity in the 7.5% gel. Solubilized membranes from erythrocytes which had been incubated 60 min with ^{14}C-labeled HN3 were subjected to gel-electrophoresis on a 7.5% gel in SDS as shown in Fig. 3C. The slab-gel was sliced vertically in 10 mm pieces and those horizontally in 3 mm pieces and solubilized in toluene/Soluene 350 as described in Materials and Methods section. The position of bands staining with Coomassie Blue or by the PAS procedure is drawn schematically on the upper part of the graph.

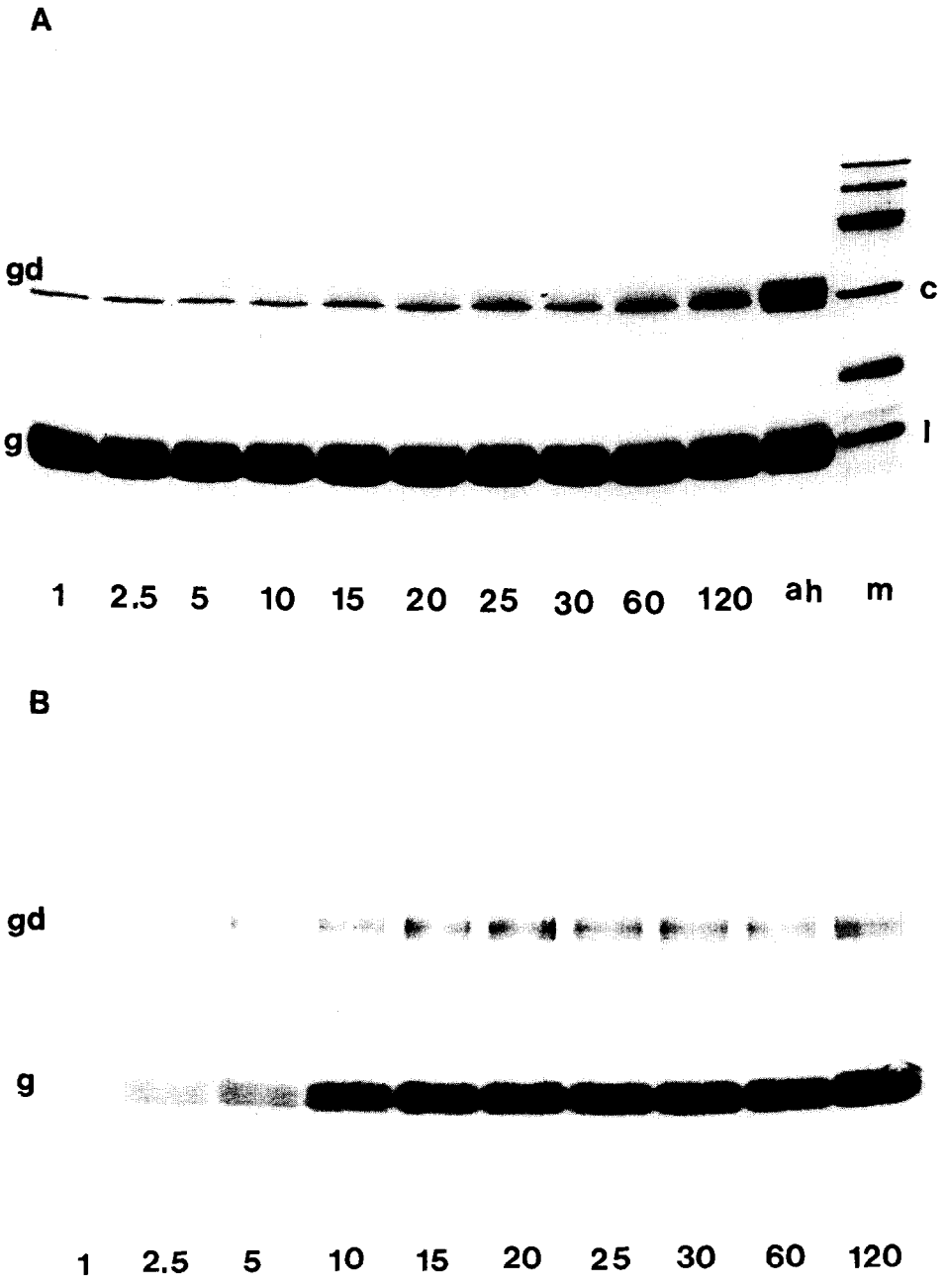

Fig. 5. 15% SDS-polyacrylamide-gel-electrophoresis of hemolysate from erythrocytes reacted with HN3. Erythrocytes were incubated with 1 mM ^{14}C-labeled HN3 at 37° and lysed by treatment with hypotonic phosphate buffer pH 8 as described in Materials and Methods section. 5 μl aliquots of a 1:1 mixture of the lysate with solubilization buffer were applied to the gel. The numbers on the bottom of the gels represent different time points. g = globin subunits; gd = globin-dimer; ah = alkylated hemoglobin (purified hemoglobin was treated with HN3 *in vitro* according to Albrecht *et al.* [8]); m = marker proteins (l = lysozyme, m.wt = 14,300); c = carbonic anhydrase, m.wt = 30,000). A: Coomassie Blue stained gel; B: autoradiograph of the dried gel.

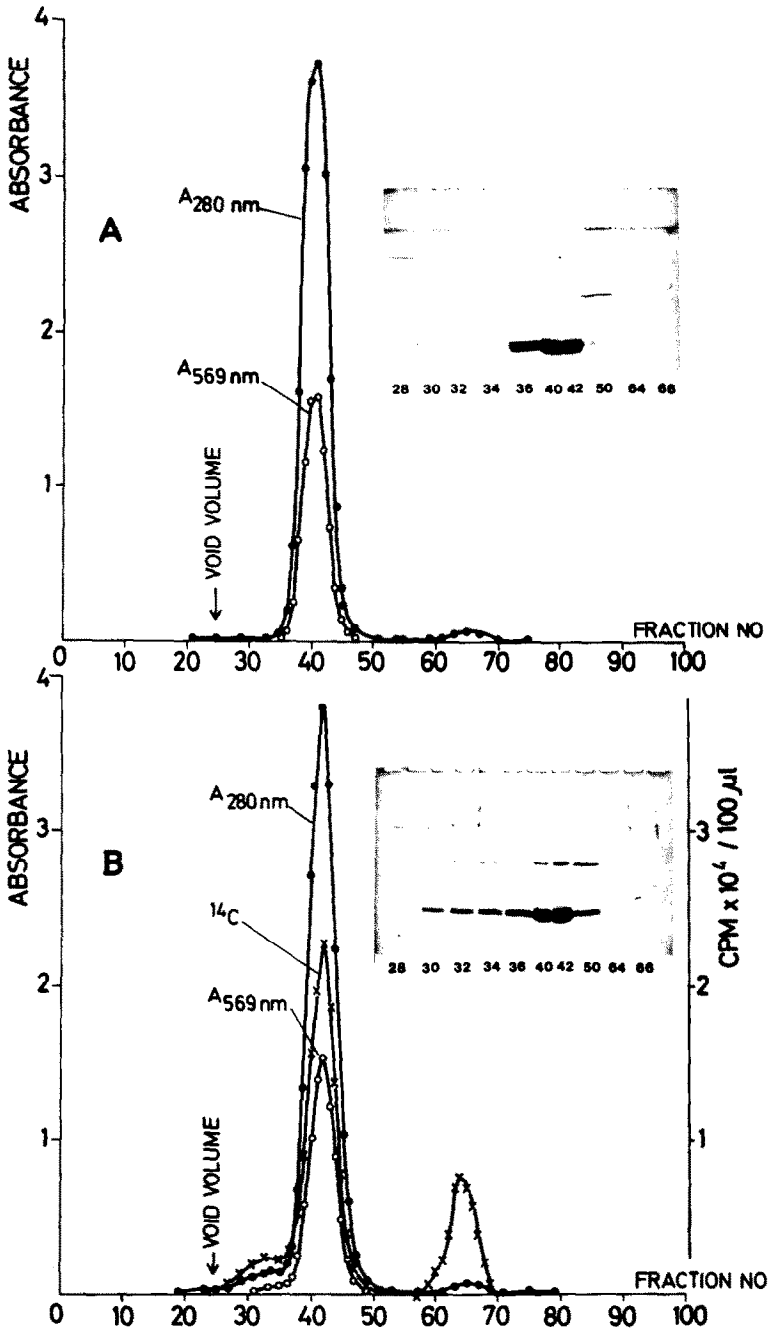

Fig. 6. Chromatography of the lysate of untreated (A) and alkylated (B) erythrocytes on a Sephadex G 200 column. 5 ml of the lysate from erythrocytes incubated for 60 min with (B) and without (A) 1 mM ^{14}C-labeled HN3 was applied to a 100 cm × 2 cm column and eluted with a 5 mM phosphate buffer pH 8.0. Fractions of 2.7 ml volume were collected. The void column was determined using Dextran Blue. Colored fractions were reacted with carbon monoxide and the absorbance at 569 nm determined. Insert: 15% SDS-gel-electrophoresis of fractions indicated by number on the bottom of the gels.

the lysate was applied to a Sephadex G 200 column and eluted with a 5 mM-phosphate buffer (Fig. 6). The bulk (74%) of the radioactivity applied to the column was found in a peak which was identified by absorption and SDS-PAGE as hemoglobin. In contrast to the lysate of untreated erythrocytes (Fig. 6A) the hemoglobin fractions from the lysate of HN3 alkylated erythrocytes contained covalently linked globin dimers as detected by SDS-PAGE (Fig. 6B, inset). Since the position of the hemoglobin peak had not changed the formation of dimers must have occurred by intramolecular cross-linking between two globin subunits. A small peak migrating ahead of the main peak consisted also of globin and globin dimers as was shown by SDS-PAGE (Fig. 6B). Since the material in the fractions comprising this peak shows a hemoglobin absorption spectrum, it was assumed to be the product of intermolecular cross-linking between two hemoglobin molecules.

16 Per cent of the applied radioactivity was found in fractions containing low molecular weight compounds not detectable by SDS-PAGE (Fig. 6B, inset). These fractions have not yet been further analysed.

The specific activity of the hemoglobin peak was calculated and found to be 5.3 nmoles/mg (Table 1).

DISCUSSION

Using the human erythrocytes as a model, the reaction of an alkylating antitumor agent with membrane proteins and cytoplasmic constituents was demonstrated on the molecular level. Following SDS-polyacrylamide-gel-electrophoresis of solubilized membrane proteins isolated from ^{14}C-labeled HN3-treated erythrocytes, several targets were detected in the membrane. By analysis of the hemolysate it was shown that HN3 also penetrates the membrane, reacting with cytoplasmic components, and in particular with hemoglobin.

The uptake of HN3 by erythrocytes is influenced by the high reactivity of the compound which reacts with components of the incubation medium as well. This causes a fast decrease in the reagent and, as shown in Fig. 1, a slowing of the uptake. Therefore, the kinetics of uptake shown in Fig. 1 do not represent saturation. Increasing the concentration of the alkylating agent in the incubation medium results in a higher level of incorporation into the erythrocytes; however, the rate of hemolysis is also increased. Thus, it was not possible to determine the HN3 concentration necessary for saturation of all possible binding sides. Figure 1 shows also the rate of uptake of hydrolysed HN3. This suggests that a part of the radioactivity found in the cells was due to the uptake of the hydrolysed compound.

The specific activities were calculated for the total membrane protein and for hemoglobin, which was separated from the other cytoplasmic components by gel-chromatography as shown in Fig. 6. Comparison of the value for total HN3-labeled membrane protein (19.3 nmoles/mg) with the specific activity of HN3-labeled hemoglobin (5.3 nmoles/mg, Table 1), suggests that the membrane is the primary target for the alkylating action of HN3 on the human erythrocyte. For a final conclusion, however, the specific activities of the single alkylated membrane proteins must be determined: a difficult task because of the problems involved in the separation of the cross-linked products.

Spectrin, which seems to be one of the first targets of the alkylating agent, is described as a peripheral membrane protein located on the cytoplasmic side of the erythrocyte membrane [16, 17]. It contains two components which separate on a SDS-polyacrylamide-gel into the bands 1 and 2 [16, 17]. It is thought to form together with actin (band 5) a network responsible for cell-shape [16, 30, 31] and is supposed to be involved in transmembrane effects [31]. Ji and Nicolson [32] showed that spectrin in ghosts becomes accessible to cross-linking by certain agents only after rearrangement following the binding of lectin. Sheetz and Singer [33] found that a structural effect on the cell-shape produced by ATP is promoted by the cross-linking of spectrin by its antibody. Whether the cross-linking observed in this study affects shape or shape-changes of erythrocytes and influences the transmembrane control of receptor binding sides remains to be investigated.

Chemical cross-linking and identification of the cross-linked products could be an approach to determine the nearest neighbours of the membrane proteins and thus give information about the topology and organisation of the membrane components [34]. However, the interpretation of cross-linking studies in relation to membrane structure is hampered by the possibility that the reagent itself causes effects on organisation and topology of the membrane components. From experiments with red cell ghosts (not shown) it was concluded that HN3 crosslinks hemoglobin to spectrin. Analysis of other products, however, will be extremely difficult, since the linkage formed by HN3 is not easy to cleave, unlike that of dimethyl-3,3′-dithiobispropionimidate dihydrochloride, which was used by Wang and Richards [21] to cross-link membrane proteins in intact human erythrocytes.

Using the PAS staining procedure for detection of glycoproteins, no cross-linking of carbohydrate-containing proteins could be visualized. The autoradiograph (Fig. 3), however, shows that ^{14}C-labeled HN3 is incorporated into glycophorin (PAS I) to a fairly high amount. Glycophorin is an antigenic protein which spans the membrane and is the receptor for certain lectins [16]. Since the covalent structure is known [35], it would be of interest to determine the side of reaction with the alkylating agent.

Rather surprising is the low yield of labeled band 3 protein, the other protein which spans the membrane. Band 3, a globular protein accessible from both sides of the membrane, has a molecular weight of about 100,000 and may form a dimer in the membrane. It is thought to be the anion channel as well as being involved in other transport systems [36,37]. In comparison with glycophorin, which accounts for 6.7 per cent of the membrane proteins [17], band 3, which comprises 24 per cent, is poorly alkylated, although there could be cross-linking to other proteins yielding products with higher molecular weight. It is also possible that this protein possesses a smaller number of groups which react with the alkylating agent under the present experimental conditions. Furthermore, the reactive groups of the protein might be buried and thus not accessible to the alkylating agent.

The intention of this work was to study the reactions

of the alkylating antitumor agent HN3 with membrane components on the molecular level, using the human erythrocyte as model. Since the erythrocyte, though sharing some features in common with other mammalian cells, is a unique cell type, the observed results cannot be generalized to explain the mechanism of antitumor action. The basic information, however, that an alkylating agent reacts with membrane proteins and modifies proteins such as spectrin, which are important for the cytoskeleton and transmembrane control, may contribute to an understanding of their action.

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