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Interactions between Cyclodextrins and Cell-Membrane Phospholipids

J. Szejtli

Biochemical Research Laboratory of Chinoin Pharm.-Chem. Works, Budapest, 1026, Endrődi S. u. 38/40, Hungary

T. Cserháti

Plant Protection Institute of Hungarian Academy of Sciences, Budapest, 1022 Hermann Ottó u. 15, Hungary

and

M. Szögyi

Institute for Biophysics, Semmelweis Medical University, Budapest, 1088 Puskin u. 9, Hungary

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SUMMARY

The interactions between cyclodextrins (CDs) and selected cell membrane phospholipids, liposomes and human erythrocytes were studied. Non-methylated cyclodextrins did not influence the differential scanning calorimetric behaviour of phospholipids and did not increase the permeability of dipalmitoyl-phosphatidyl-choline liposomes. Dimethyl- and trimethyl- β-CD interacted with the phospholipids but the effect was negligible compared to the effect of valinomycin. Reversed-phase thin-layer chromatography revealed complex formation with dimethyl-β-cyclodextrin, but not with others. The addition of cyclodextrins up to 10^{-2} *mol litre*$^{-1}$ *concentration did not modify the active or passive alkali ion transport of human erythrocytes, however, higher concentrations of added β-cyclodextrin especially dimethyl-β-cyclodextrin resulted in hemolysis.*

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INTRODUCTION

Cyclodextrins form inclusion complexes with guest molecules of an appropriate size and polarity, modifying their stability, solubility and volatility (Bender & Komiyama, 1978; Szejtli, 1982*b*). The stability, water-solubility and hence bioavailability of many drugs is considerably improved by complexing with cyclodextrins. (Szejtli 1982*a*). Extensive toxicological (Makita *et al.*, 1975; Szejtli & Sebestyén, 1979; Szejtli, 1982*a*) and metabolic (Andersen *et al.*, 1963; Gerlóczy *et al.*, 1985) studies have been performed with β-cyclodextrin. Despite the rapidly increasing number of papers (Pitha *et al.*, 1983) that have described the advantages of complexing drugs with cyclodextrins, the marketing of cyclodextrin-containing drugs is expected only in the second half of the 1980s. Flavours stabilized by β-cyclodextrin are already on the market in Hungary. In Japan, where cyclodextrins are considered as enzymatically modified starches, β-cyclodextrin is used in many food products, e.g. in aspartame (non-nutritive sweetner) tablets (Ojima *et al.*, 1984). Because of its price and availability, only β-cyclodextrin, which is produced already on industrial scale, can be considered for use in foods over the next decade. In the drug industry however the use of α-, γ- and methylated cyclodextrins is also expected.

The various cyclodextrins show surprising and unexpected differences in those properties which are most important for any potential use, Table 1 illustrates some characteristic data. The solubilities show particularly interesting differences. According to animal tests, the absorption of intact β-cyclodextrin molecules from the intestinal tract is negligible (Gerlóczy *et al.*, 1985). Its metabolism is attributed to the intestinal microflora in the colon (Antenucci & Palmer, 1984). Therefore no toxic symptoms were expected following oral administration even of extremely high doses. However parenteral administration results in nephrotoxicity (Frank *et al.*, 1976). In view of the low water-solubility of β-cyclodextrin, and especially of its complexes, and its high resistance to amylolytic degradation this parenteral toxicity is not surprising. It can be seen from Table 1 that the other CDs (α- and γ-CD, and methylated β-CDs) behave differently in just these properties. It therefore seems probable that γ-CD is rapidly degraded in the bloodstream, and thus will have no adverse effects on the kidneys (Matsuda *et al.*, 1983). α-Cyclodextrin is unlikely to be degraded in the bloodstream but because it has the smallest cavity diameter its applicability is

TABLE 1
Some Characteristics of Cyclodextrins

	α-CD	*β-CD*	*γ-CD*	*DIMEB*[a]
Number of glucopyranosidic residues	6	7	8	7
Molecular weight	972	1 135	1 297	1 331
Cavity diameter (nm)	0·47-0·6	0·8	1·0	0·8
Solubility in water (mg ml^{-1})				
at 25°C	145	18	232	500
at 80°C	–	196	–	5
V_{max} (min^{-1}) of hydrolysis (Jodál *et al.*, 1983) with *Aspergillus oryzae* α-amylase at pH 5·2, 37°C	5·8	166	2 300	0
Concentration in mg ml^{-1} causing erythrocyte hemolysis at 37°C (Irie *et al.*, 1982*a*)	5·7	3·5	21	
Solubility of oestrone[b] at 25°C in 0·075 M CD solutions (Habon *et al.*, 1985)	0·06	0·19[c]	0·35	4·75

[a] Heptakis (2,6-di-O-methyl) β-cyclodextrin.
[b] Oestrone solubility in water is 0·03 mg ml^{-1}.
[c] β-CD concentration was 0·0112 M.

rather restricted. DIMEB (heptakis-(2,6-di-O-methyl)-β-cyclodextrin) probably cannot be metabolized and is excreted rapidly through the kidneys (Szejtli, 1983), but because of its 14 methoxy-groups it is expected to behave differently towards cell membranes than the non-substituted cyclodextrins which contain only hydroxyl groups.

Irie *et al.* (1982*a*) reported that cyclodextrins at low concentrations (5 mM for α-, and 10 mM for γ-CD) protect the human erythrocytes against osmotic and heat-induced hemolysis, while higher concentrations (above 3 mM for β-, 6 mM for α-, and 16 mM for γ-CD, at 37°C, pH 7·4 in 10 mM isotonic phosphate buffer) cause hemolysis. At these higher concentrations cyclodextrins caused the release of cholesterol from the cell membrane (in the above order: $\beta > \gamma > \alpha$-CD), which indicates that the CD-induced hemolysis is a secondary event, resulting

from the interaction of CDs and membrane components. Scanning electron microscopy studies suggest that the protective effects of CDs below the critical concentrations mentioned may be ascribed to membrane expansion. Probably the CDs alter the mechanical properties of the membrane lipids, protecting the erythrocytes, but at higher CD concentrations these lipids are sequestered from the membrane, resulting in its disruption.

The protective effect can be utilized in parenteral administration of drugs inducing hemolysis or local irritation. Tiamulin (Sato *et al.*, 1982), imipramine (Uekama *et al.*, 1983), chlorpromazine, flufenamic acid (Uekama *et al.*, 1981*a*, 1981*b*, 1982) and phenothiazines (Irie *et al.*, 1982*b*, 1983) have all been studied recently: cyclodextrins were shown to be effective agents in reducing the drug-induced hemolysis. In these cases probably the reduction of the non-complexed, free drug concentration was the decisive factor since the concentration of the CDs was rather low. A good correlation between the stability constants of drug–CD complexes (with different CDs) and the inhibitory effects on drug-induced hemolysis was found.

For a parenterally administered drug preparation (injection) containing cyclodextrin, the crucial question is 'what interactions can be expected between the cyclodextrin and the cell membranes?' Interactions between cyclodextrins and lipids (Szejtli, 1982*b*) and erythrocytes (Irie *et al.*, 1982*a*) have been reported. Cyclodextrins also affect the biosynthesis of lipids (Bergeron *et al.*, 1975), the hydrolysis of glycerides, and fat absorption (Szejtli *et al.*, 1985).

Phospholipids play a fundamental role in the dynamic and structural properties of cells membranes (Singer, 1981, 1982). The correlation between structural and functional changes in lipid layers is supposed to be similar in *in vivo* biological and in *in vitro* model membranes. Therefore the study of interactions between liposomes or phospholipids and potential cell membrane damaging agents provides information about the expected biological effects (Roman & Keana, 1982). The present paper deals with interactions between cyclodextrins, and cell-membrane phospholipids.

MATERIALS AND METHODS

α-, β-, and γ-cyclodextrins are marketed by Chinoin, Budapest: DIMEB (heptakis (2,6-di-O-methyl)-β-cyclodextrin) and TRIMEB (heptakis

(2,3,6-tri-O-methyl)-β-cyclodextrin) have been prepared in the Biochemical Research Laboratory of Chinoin as described by Szejtli *et al.*, 1980).

The cyclodextrins were chromatographically pure except DIMEB which showed a slower moving secondary minor spot on TLC in addition to the intensive main spot.

Dipalmitoyl-phosphatidyl-choline (DPPC), dioleoyl-phosphatidyl-choline (DOPC) and dipalmitoyl-phosphatidyl-ethanolamine (DPPE) were purchased from the Sigma Chemical Co. and were used without additional purification.

Differential scanning calorimetry

Dry lipids were vigorously mixed with twice-distilled water (weight ratio 1:4) and with cyclodextrins (molar ratio 1:10) at 50°C for 30 min in a vortex mixer. The measurements were carried out on a Dupont 990 Thermal Analyzer at a heating rate of 5°C min^{-1}. Due to the low main transition temperature of DOPC (−22°C) it was not included in our DSC and permeability investigations.

Determination of permeability of liposomes

Liposomes were formed by sonification from DPPC in 0·16 M KCl solution containing tracer amounts of ^{42}KCl. After overnight equilibration the lipid suspension was passed down a column of Sephadex G-50 (1·5 × 30 cm) to remove the excess tracer not trapped within the liposomes. The liposomes were eluted from the column with 0·16 M KCl solution (flow rate 0·5 ml min^{-1}): 3 ml portions of eluted liposomes were dialyzed against 10 ml of 0·16 M KCl solution. The efflux rate was measured for consecutive 15-min periods. At the end the ^{42}K content of liposomes was measured using a gamma scintillation counter. The cyclodextrins being investigated were added to the liposomes in a molar ratio of 1:100. The membrane-damaging effect of cyclodextrins was compared with valinomycin, a well known membrane disorganizing compound. The latter was used in a molar ratio of 1:200 because at a 1:100 ratio it totally disorganized the liposomes. The permeability constants were calculated according to Johnson & Bangham (1969). DPPE was not suitable for the formation of liposomes using this method.

Reversed-phase thin-layer chromatography

DC-Alufolien Cellulose (Merck) plates were impregnated by overnight immersion in a solution of 5% paraffin oil in *n*-hexane. The phospholipids were dissolved in chloroform at a concentration of 5 mg ml^{-1}, 2 ml of each solution were spotted on the plates. To improve reproducibility the method of Cserháti & János (1982) was applied. Water and ethanol, and water and *n*-propanol mixed in various volume ratios were applied as eluents. After development the plates were dried and the spots were detected by iodine vapour, Dragendorff reagent or by ninhydrin.

Cyclodextrins were dissolved in the eluent in the concentration range of 1–50 mg ml^{-1} eluent. The $R_M = \log((1/R_f) - 1)$ values determined in an eluent free of cyclodextrin served as controls. For each experiment five independent parallel determinations were made.

Transport of radioactive cations through the cell membrane of human erythrocytes at 37°C

Coagulation-inhibited human blood was stored for 1–2 days at 5°C, centrifuged and washed 3 times with artificial serum (composition in mmol $litre^{-1}$: NaCl, 138; KCl, 4·1; $MgCl_2$, 2·5; $CaCl_2$, 1; glucose, 2·7). The washed erythrocytes were suspended in a volume of artificial serum that corresponded to the plasma-volume of the original blood, and the ^{42}K or ^{86}Rb isotopes (2–5 MBq) were admixed to this suspension. The concentration of cyclodextrins in the extracellular volume was 10^{-6}–10^{-2} mol $litre^{-1}$, and they were dissolved in the artificial serum before suspending the erythrocytes. This suspension was incubated at 37°C, samples were taken at corresponding time intervals, and after centrifugation the radioactivity of the extracellular solution was determined. The active transport, i.e. the uptake of radioisotopes by the erythrocytes was followed by determining the decrease in radioactivity of the extracellular solution, and expressed as a percentage of the original radioactivity.

Release of radioactive cations from human erythrocytes at 20°C

Human blood was incubated for 2·5 h at 37°C with $^{42}K^+$, $^{86}Rb^+$ or $^{137}Cs^+$. After centrifuging, the erythrocytes were washed and suspended, as before, in artificial serum. The cyclodextrins were dissolved in this

suspension, and these were incubated at 20°C with careful stirring. (The active transport is negligible at this temperature.) Samples were taken, centrifuged, and the radioactivity of the extracellular solution determined. The amount of released radioactivity was expressed as a percentage of the total radioactivity of the erythrocytes.

RESULTS AND DISCUSSION

Differential scanning calorimetry (DSC) has been successfully applied to study the interaction of phospholipids with a great number of membrane-damaging agents. Disappearance of the pre-transition peak, modification of the energy of the main transition and its temperature indicate the disruption of the highly organized structure built from the fatty acid chains of phospholipids and their polar head groups.

Cyclodextrins exerted no effect on the DSC behavior of DPPC and DPPE, that is no stable cyclodextrin inclusion complex was formed with these phospholipids. DIMEB and TRIMEB did not modify the main transition temperature of DPPE but they decreased slightly the main transition temperature of DPPC (Table 2).

Cell membrane-damaging substances that interact with phospholipids would result in much greater DSC behavior effects. Because the fatty acid chains are identical, the difference between the behavior of DPPC and DPPE suggests that the polar head groups play a role in complex formation.

The addition of membrane disorganizing agents to phospholipid liposomes strongly increases their permeability (e.g. efflux of included

TABLE 2

Effect of Methylated CDs on the Main Transition Temperature of Dipalmitoyl-phosphatidyl-choline (DPPC) and on the Membrane Permeability of DPPC liposomes

	Transition temperature (°C)	*Permeability constant (s^{-1})*
Control	41·5	$0{\cdot}26 \times 10^{-4}$
DIMEB	41·0	$0{\cdot}55 \times 10^{-4}$
TRIMEB	40·5	$1{\cdot}63 \times 10^{-4}$
Valinomycin	–	$11{\cdot}20 \times 10^{-4}$

radioactive cations). When a substance in *in vitro* tests results in an increased permeability of a phospholipid liposome then cell membrane damage *in vivo* is very probable. The permeability data parallelled the results obtained by DSC measurements. The unsubstituted cyclodextrins did not increase the permeablity of DPPC liposomes but the methylated derivatives slightly increased the permeability (Fig. 1). (The permeability constants are shown in Table 2.)

As the outer surface of the cyclodextrin molecules is hydrophilic, and on complex formation the hydrophobic part of the phospholipid molecules will be inserted into the apolar cavity of the cyclodextrin, complex formation results in decreased lipophilicity of the guest molecule. This decrease in lipophilicity can be determined by reversed-phase thin-layer chromatography (RPTLC) (Biagi *et al.*, 1969; Boyce & Milborrow, 1965.). A larger difference between the R_M values (unit of lipophilicity in RPTLC) of a compound determined in the presence or absence of a cyclodextrin in the eluent means greater complex stability, i.e. a stronger interaction between the interacting substances (Cserháti *et al.*, 1983*a*). This principle has been successfully applied to the study of cyclodextrin complex formation with a series of compounds, e.g. polymixine (Cserháti *et al.*, 1983*b*), triphenylmethane derivatives, nitrostyrenes and substituted *s*-triazine derivatives (Cserháti *et al.*,

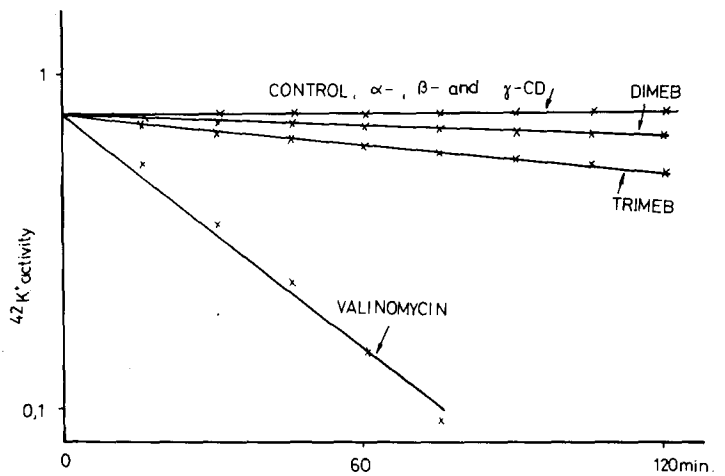

Fig. 1. Potassium efflux from DPPC liposomes. Molar ratio of α-, β-, γ-CD, DIMEB and TRIMEB to phospholipids 1 : 100 and to valinomycin, 1 : 200.

1983*d*). As the stability of cyclodextrin inclusion complexes is greater in more polar solvent mixtures (Cserháti *et al.*, 1983*b*.) the most polar eluents possible were selected for the RPTLC investigations. Because the adsorptive character and surface pH value of a support influences the retention of polar substances even after impregnation (Cserháti *et al.*, 1983*c*), the most polar cellulose has been chosen as the support in our investigations. Compared with the silica and alumina more frequently used as RPTLC supports, cellulose is characterized by a lower energy of adsorption and a neutral surface pH value.

The saturated fatty acid derivatives DPPE and DPPC showed extremely elongated spots in all RPTLC systems. It is difficult to evaluate this type of spot quantitatively. However, it could be seen that DIMEB and TRIMEB reduced the lipophilicity of DPPC but not that of DPPE. The unsaturated DOPC gave regular spots in eluents containing 40–60% ethanol. No reduction in lipophilicity of DOPC by α-, γ-cyclo-

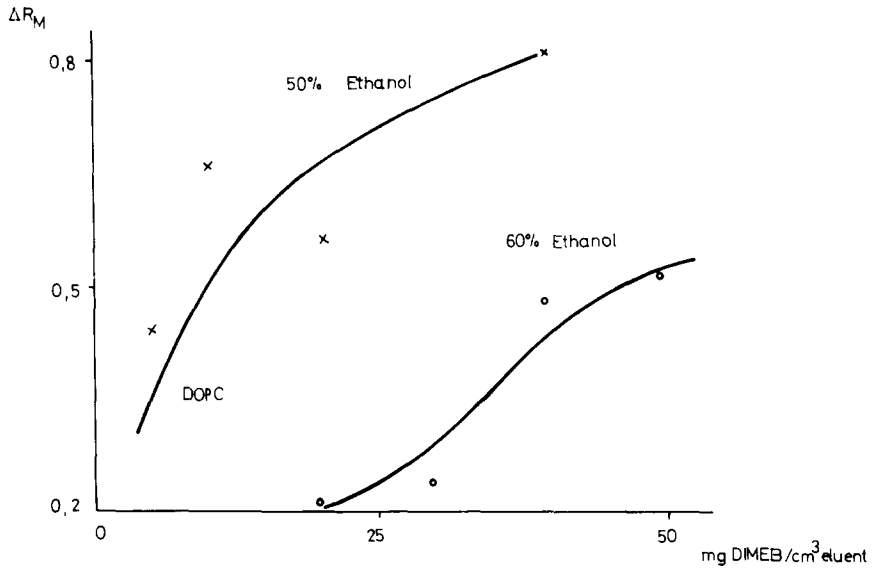

Fig. 2. Decrease in the lipophilicity of DOPC with increasing DIMEB concentration at different ethanol concentrations.

$$\Delta R_M = R_{M1} - R_{M2}$$

R_{M1} = lipophilicity value of DOPC determined in eluents containing different quantities of DIMEB. R_{M2} = lipophilicity value of DOPC in DIMEB-free eluent.

dextrins and TRIMEB was observed at this ethanol concentration, even at an eluent concentration of 50 mg ml^{-1}; β-cyclodextrin was also ineffective (due to the low solubility its highest eluent concentration was only 13 mg ml^{-1}). DIMEB, however, reduced the lipophilicity of DOPC (Fig. 2). This finding can be explained again by assuming that the lipophilic part of DOPC (the hydrophobic fatty acid chains) is inserted into the cavity of DIMEB resulting in elevated hydrophilicity. The effect was higher at lower ethanol concentration (Fig. 3). These data prove again that decreasing the dielectric constant of the solvent and/or increasing the concentration of competing guest molecules (e.g. higher ethanol concentration) lowers the stability of the inclusion complexes.

Methylated cyclodextrins (especially TRIMEB) are more lipophilic than the non-substituted cyclodextrins, therefore in addition to the inclusion-type interaction, hydrophobic substructures of phospholipids might occur. When complexing a fatty acid mixture with β-CD, an

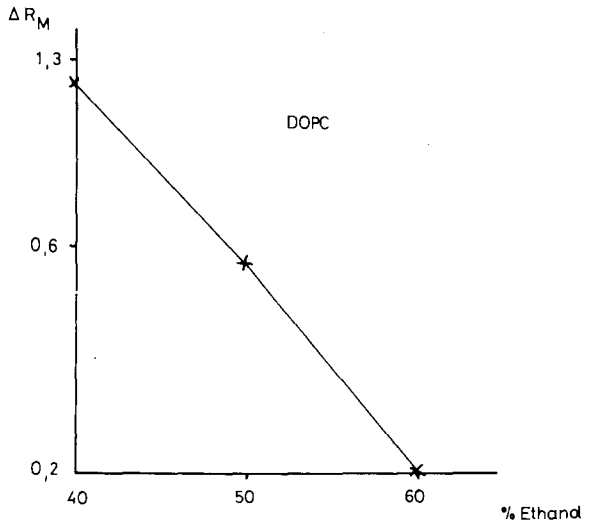

Fig. 3. Effect of the solvent dielectric constant on the inclusion complex stability of DOPC.

$$\Delta R_M = R_{M1} - R_{M2}$$

R_{M1} = lipophilicity value of DOPC determined in eluents containing 20 mg DIMEB cm^{-3}. R_{M2} = lipophilicity values of DOPC in DIMEB-free eluent.

enrichment in unsaturated fatty acids was observed in the complexed fraction (Szejtli *et al.*, 1979), this shows that the unsaturated fatty acids are better complex forming partners.

Also in this case, the unsaturated chains of DOPC must have different steric requirements to fit the cyclodextrin cavity as compared with the saturated fatty acids: presumably only DIMEB is able to comply with the steric requirements. The TRIMEB cavity is already too narrow on account of the permethylated structure. The decisive proof of an interaction between cyclodextrins and cell membranes comes from the study of the uptake and release of cations by human erythrocytes at 37°C and 20°C, respectively. The cation transport through the living cell membrane at 37°C is a facilitated translocation, i.e. active transport, while at 20°C this complex transport mechanism becomes paralyzed, and the observed cation efflux is

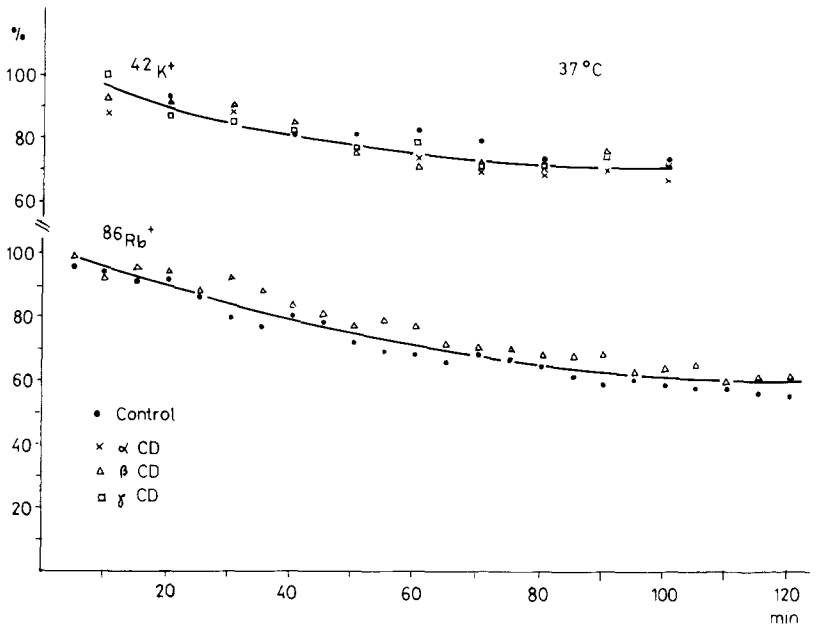

Fig. 4. Decrease in the radioactivity of extracellular solutions through uptake of ^{42}K and ^{86}Rb cations by human erythrocytes by active transport at 37°C: the upper curve, 10^{-2} M α-(x), β-(△), γ-(□) cyclodextrins and without cyclodextrins (●); lower curve, $1{\cdot}7 \times 10^{-3}$ M β-CD (△) and control containing no cyclodextrins (●).

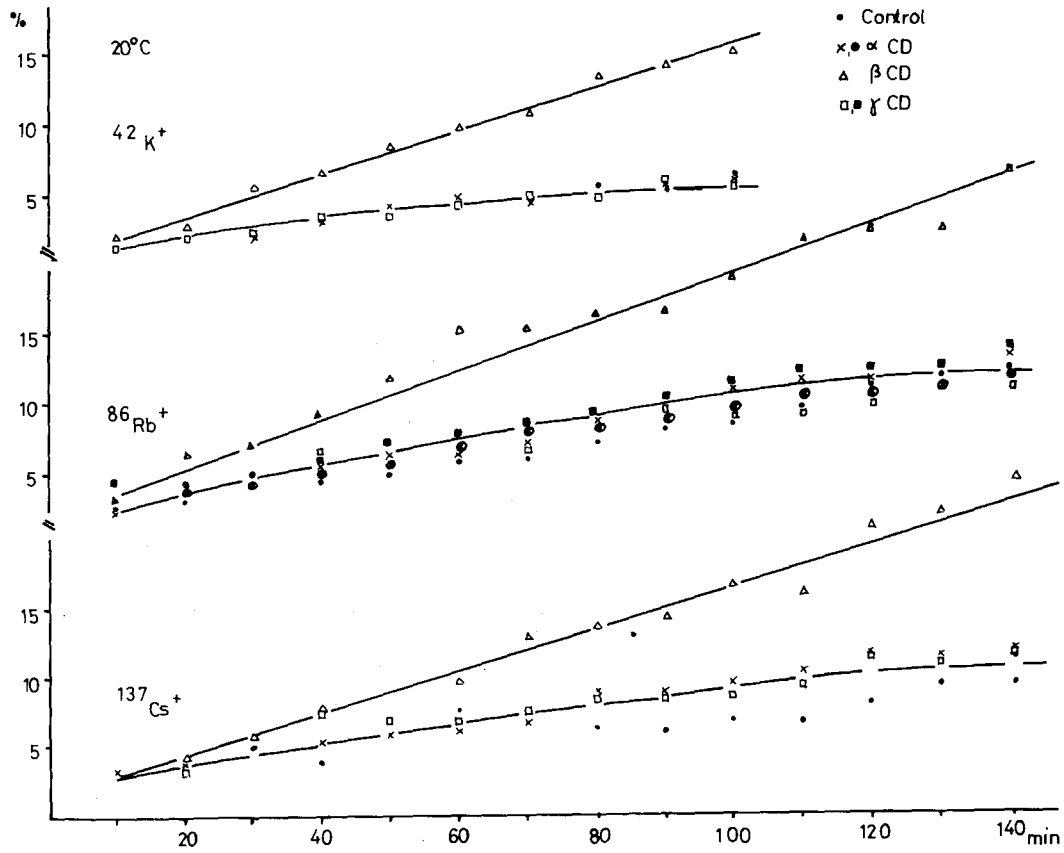

Fig. 5. Release of radioactive alkali cation from human erythrocytes by passive transport at 20°C: upper curve, ^{42}K, control (●), α-(x), β-(△), γ-(□) CD, all at $1{\cdot}7 \times 10^{-2}$ M; middle curve ^{86}Rb, α-CD $1{\cdot}7 \times 10^{-3}$ M (x) and $1{\cdot}7 \times 10^{-2}$ M (⊕), β-CD $1{\cdot}7 \times 10^{-2}$ M (△), γ-CD $1{\cdot}7 \times 10^{-3}$ M (■) and $1{\cdot}7 \times 10^{-2}$ M (⊡); lower curve, ^{137}Cs, control (●), α-(x), β-(△), γ-(□) CD, all at $1{\cdot}7 \times 10^{-2}$ M.

attributed to a passive diffusion (passive transport) (Gárdos, 1972; Sarkadi *et al.*, 1982; Skou, 1975; Whittam, 1975). In the present work the active transport is characterized by the rate of uptake of ^{42}K or ^{86}Rb ions, at 37°C, from the extracellular solution. Figure 4 illustrates that the non-substituted cyclodextrins (at concentrations up to 10^{-2} mol litre^{-1}) do not influence the active cation transport into the erythrocytes. DIMEB and TRIMEB behave similarly. All points in Fig. 4 represent the average of 4 replicates. At cyclodextrin concentrations of up to 10^{-2} mol litre^{-1} no hemolysis was observed.

Similarly, passive transport studies at 20°C showed no membrane damaging effect at concentrations up to 10^{-2} mol litre^{-1}; however, β-cyclodextrin concentrations of $1{\cdot}7 \times 10^{-2}$ mol litre^{-1} resulted in a 8–10% hemolysis and DIMEB resulted in a complete hemolysis. Figure 5 illustrates the release (passive permeability) of the three radioactive cations from human erythrocytes in the presence of $1{\cdot}7 \times 10^{-2}$ mol litre^{-1} of cyclodextrins.

No enhancement of passive transport was observed in the presence of α- and γ-cyclodextrins, but β-cyclodextrin caused a significant increase. The effect of DIMEB could not be determined because it caused a complete hemolysis. The fact that cyclodextrins up to a concentration of 10^{-2} mol litre^{-1} did not modify the active or passive alkali ion transport of human erythrocytes, leads us to suppose that they result only in reversible modifications in the membrane structures, or in the function of the transporting mechanism. At higher concentrations cyclodextrin addition, particularly DIMEB, causes hemolysis.

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