Alterations in the Activities of Rabbit Erythrocyte Membrane-Bound Enzymes Induced by Cholesterol Enrichment and Depletion Procedures

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Z. Naturforsch. **41c,** 301–309 (1986); received October 25, 1985

(Na^+,K^+)-Stimulated ATPase, Acetylcholinesterase, NAD^+ase, Cholesterol, Rabbit Erythrocyte Membrane

The effect of cholesterol enrichment and depletion of rabbit erythrocytes on the activities of membrane-bound enzymes, namely (Na^+,K^+)-stimulated ATPase, NAD^+ase and acetylcholinesterase was examined.

The cholesterol content of erythrocyte membranes has been modified by incubation of intact cells with sonicated egg lecithin/cholesterol vesicles (cholesterol/phospholipid molar ratio approx. 2) and with egg lecithin vesicles for time intervals up to 10 hours. The cholesterol/phospholipid molar ratio (CH/PL) of untreated rabbit red blood cell membranes was 0.92–0.94. Linear increase (up to CH/PL molar ratio 1.72–1.9) or decrease (up to CH/PL molar ratio 0.27–0.43) in cholesterol content of erythrocyte membranes was observed over the 10 hours of incubation with egg lecithin/cholesterol and egg lecithin liposomes respectively. Fusion of liposomes to the membrane or their attachment to the membrane surface was not a significant factor in the alteration of CH/PL ratio. (Na^+,K^+)-stimulated ATPase, NAD^+ase and acetylcholinesterase activities were measured as a function of membrane cholesterol. The specific activities of all three enzymes were progressively decreased with increase in cholesterol content. Partial reversibility of the inhibitory effect of cholesterol was demonstrated by measurements on cells depleted again after cholesterol enrichment. This was confirmed by the fact that a lowering in cholesterol content evoked an analogous activation of enzymes. The possible implications of physicochemical modifications of bulk and annular lipids of membrane-bound enzymes in the inhibition mechanism are discussed.

Introduction

The function and architecture of the red cell membrane is mainly regulated by the composition of lipids and proteins [1]. It has been shown that changes in red cell lipids take place in the circulation through interactions with altered plasma lipids [2, 3]. Red cell cholesterol is in free equilibrium with unesterified plasma cholesterol which in turn is regulated by the enzyme lecithin:cholesterol acyl transferase (LCAT) [4]. Thus, changes in various biochemical parameters of blood plasma as a result of disease or diet as well as modifications of lipid metabolism by hormones may lead to alterations in cholesterol content of erythrocytes.

It has been observed that cholesterol from either very dilute aqueous solutions, in which it is present in a monomeric form, or from lipid vesicles (liposomes) can be incorporated into the structure of various biomembranes causing deep functional changes of all studied integral proteins [5–10]. Such phenomena have been recently verified by *in vivo* experiments [11, 12]. Alterations of the cholesterol content can change the fluid nature of these membranes and perhaps exert, a constraint upon enzyme and transporter activity [13, 14].

The purpose of this study was to look at the effect of membrane cholesterol on the activity of (Na^+,K^+)-stimulated ATPase, acetylcholinesterase and nicotinamide adenine dinucleotide splitting enzyme (NAD^+ase) of erythrocyte membrane. The data concerning the effect of cholesterol on the activity of (Na^+,K^+)-stimulated ATPase are rather conflicting [15] although this enzyme is strongly related to the osmotic equilibrium and energy consumption by erythrocytes. Physiological function of externaly oriented enzymes NAD^+ase and acetylcholinesterase in red blood cell membrane remains unknown and the possibility that the last activity is picked up from the plasma rather than originated within the erythrocyte is discussed [16]. The possibility of correlations between the enzymatic activity of NAD^+ase and acetylcholinesterase and cholesterol content of erythrocyte membrane was examined to show how deep these enzymes are embedded into the lipid matrix of

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0341–0382/86/0300–0301 $ 01.30/0

membrane. Besides, acetylcholinesterase and (Na^+,K^+)-ATPase seems to be a few among the recognized erythrocyte plasma membrane enzymes to exhibit activity changes in some haematologic disorders [17, 18] and the mechanism of these changes is yet to be defined.

It is shown that profound functional changes are induced in erythrocyte membrane proteins by exogenous cholesterol. The reversibility of the cholesterol-induced changes served as a criterion for the causal involvement of cholesterol in the effects observed.

Materials and Methods

Materials

Blood was obtained from male albino rabbits 5–8 months old after overnight fasting and was anticoagulated with heparin (10 units/ml). Cholesterol was obtained from Merck and exhibited a single spot when analyzed on thin-layer chromatography in petroleum ether/diethyl ether/glacial acetic acid (90:10:1, v/v) and visualized by acid charring. Purified egg lecithin was supplied by Sigma Chemical Co. and produced a single spot with thin-layer chromatography on silica gel G using a solvent system composed of chloroform/methanol/acetic acid/water (20:12:3:1, v/v) visualized by iodine vapor. The sodium salt of adenosine-5′-triphosphate (ATP), nicotinamide adenine dinucleotide (NAD^+) were obtained from Sigma Chemical Co. Cholesterol oxidase assay kit was from Boehringer Mannheim. Acetylthiocholine iodide and 5,5′-dithiobis-2-nitrobenzoic acid were purchased from Serva Feinbiochemica Co. All other chemicals of standard analytical grade were obtained from Merck.

Liposomal preparation

Liposomes were prepared according to Cooper *et al.* [19] with the following modification: 40 mg of egg lecithin or a mixture of 80 mg cholesterol and 40 mg egg lecithin (cholesterol/phospholipid molar ratio of 4.0) were dissolved in $CHCl_3$. The mixture was dried under a stream of N_2. The dry lipids were sonicated to clarity (30 min at 25 °C) in 100 mM NaCl/15 mM KCl/10 mM dextrose/0.1 mM Na EDTA/8 mM Tris · HCl, pH 7.4 sonication buffer. The dispersions were centrifuged at 40,000 × *g* for 1 h to remove undispersed lipids and titanium dislodged from sonicator probes. The clear supernatant was removed and used for subsequent incubation with erythrocytes. Liposomal preparations were extracted with chloroform/methanol (2:1, v/v) and the extract chromatographed on silica gel G in a solvent system of chloroform/methanol/acetic acid/water (20:12:3:1, v/v) with appropriate standard. The content of lipid phosphorous was measured by the method of Bartlett [20] in the spots after digestion with 70% $HClO_4$ at 180 °C, the cholesterol content was estimated using the reaction of Zieberman [21]. Cholesterol/phospholipid molar ratio in our liposome preparations ranged from 2.0 to 2.3.

Treatment of cells

Freshly drawn heparinized blood was centrifuged 20 min at 1,500 × *g* at 4 °C. The plasma and buffy coat were removed by aspiration. The packed red blood cells (RBCS) were washed three times with 4 volumes of ice-cold 154 mM NaCl, the buffy interfacial layer having been removed after each centrifugation. Centrifuge-packed erythrocytes were diluted (1:1, v/v) with sonication buffer and used immediately, RBC suspentions (haematocrit 10%) were incubated with cholesterol/egg lecithin liposomes, egg lecithin liposomes or with sonication buffer alone at 37 °C under gentle shaking with addition of 100 IU/ml penicillin-G and 100 μg/ml streptomycin sulphate. Aliquots of the incubation mixture were removed at appropriate time intervals. The cells were separated from residual free vesicles by centrifugation at 2,500 × *g* for 20 min at 2–4 °C. Cells were washed three times with cold 150 mM NaCl/5 mM Tris · HCl pH 7.4 and erythrocyte membranes were prepared.

Erythrocyte membrane preparation

For preparation of erythrocyte membranes the washed erythrocytes were lysed as described by Galbraith and Watts [22]. The haemolysate was centrifuged at 35,000 × *g* for 30 min with 40–60 vol. of cold 0.2 M Tris · HCl pH 7.2 three or more times until white or very pale pink. Membranes were suspended in 0.2 M Tris · HCl pH 7.2 at a concentration of 2 mg protein per ml. The protein content was determined by the method of Lowry *et al.* [23] after treatment of the membrane suspention with 1 M NaOH for 30 min. The contamined haemoglobin in membrane preparations was determined by the pyridine-hemochromogen method [24]. Erythrocyte

membrane lipids were extracted overnight by 20 vol, chloroform/methanol (2:1, v/v) according to Folch *et al.* [25]. Lipid phosphorus was determined in the aliquots of extract by the method of Bartlett [20]. Cholesterol content of erythrocyte membranes was estimated by the cholesterol oxidase method using a Boehringer assay kit. The cholesterol and phospholipids content of membranes were expressed as μmol of lipid per mg membrane protein and cholesterol/phospholipid (CH/PL) molar ratio was estimated.

Enzyme assays

(Na^+,K^+)-stimulated ATPase activity of erythrocyte membranes was assayed in an incubation medium consisting of 40 mM Tris · HCl pH 7.4, 3 mM disodium ATP, 80 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$, 1 mM ouabain and 0.15 mg of membrane protein, in a final volume of 1.5 ml. Incubations were carried out in the reaction medium for 1 h at 37 °C. The reaction was started by the addition of ATP and stopped with 0.2 ml of 50% trichloroacetic acid. After centrifugation, inorganic phosphate was determined in the supernatant by the method of Fiske and Subbarow [26]. (Na^+,K^+)-stimulated ATPase activity was defined as the difference between the inorganic phosphate liberated during incubation in the presence and absence of ouabain and expressed as μmol Pi split per mg protein per 1 h.

Acetylcholinesterase activity was determined by following the hydrolysis of acetylthiocholine iodide according to the method of Ellman *et al.* [27]. The assay mixture (3 ml) contained 20 μl of substrate (acetylthiocholine iodide, 0.075 M), 100 μl of 5,5′-dithiobis-2-nitrobenzoic acid (0.01 M in 0.1 M Tris · HCl, pH 7, and 0.02 M $NaHCO_3$), 100 mM NaCl and 10 mM Tris · HCl, pH 8. The reaction was started by the addition of 0.05 mg protein and followed spectrophotometrically by the increase in absorbance at 412 nm using a Beckman Acta MVI Spectrophotometer. The specific activity of enzyme was expressed as ΔOD per mg protein per min at 25 °C.

NAD^+ase activity was assayed essentially as described by Alivisatos and Denstedt [28], by adding 0.1 ml of 7 mM NAD^+ to the erythrocyte membrane suspention (0.2 mg protein) in a final volume 1.0 ml of 0.2 M Tris · HCl, pH 8.2. Incubations were carried out at 37 °C for 15 min with gentle shaking. Hydrolyzed NAD^+ was determined after deproteinization with 0.1 ml of 50% trichloroacetic acid, by measurement of the absorbance at 327 nm in the presence of 2 M NaCN. The enzyme activity was expressed as μmol NAD^+ split per mg protein per 15 min at 37 °C.

Results

Incubation of RBCS with either cholesterol/egg lecithin or egg lecithin liposomes usually did not modify their phospholipid and membrane protein composition and content [29]. In our experiments the amount of membrane proteins per ml of packed RBCS was the same before and after the incubation of erythrocytes with liposomes or buffer. Release of membrane-bound enzymes into the medium was not detectable during the 10 hours incubation time. This finding implies that the procedure used for modification of membrane cholesterol did not lead to loss of membrane proteins. Contaminating haemoglobin in cholesterol-enriched, cholesterol-depleted and control (buffer treated) RBCS membrane preparations, was less than 0.5% of the total membrane protein, therefore the cholesterol and phospholipid content of membranes could be expressed per mg of erythrocyte ghosts protein. No net flux of vesicle phospholipid into the red cell membrane occurred during the period in which a net flux of cholesterol either into or out the cell was observed. This is confirmed by the fact that measurement of membrane phospholipid/protein ratio from erythrocyte membrane preparations after 10 h incubation of erythrocytes with each of two kinds of liposomes showed no statistically significant difference ($P > 0.05$) between liposomes treated and control erythrocytes as determined by phosphate assay and assay by the method of Lowry *et al.* [23]. The results are expressed in terms of μmol of lipid phosphorus per mg membrane protein (control = 0.290 ± 0.020, cholesterol-enriched = 0.294 ± 0.022, cholesterol-depleted = 0.296 ± 0.025, mean ± SD for 4 samples).

Fig. 1 shows the change of the cholesterol/phospholipid (CH/PL) molar ratio in the purified RBC membranes during incubation of erythrocytes with cholesterol/egg lecithin, egg lecithin liposomes and in the same incubation medium but without liposomes. Erythrocytes took up cholesterol from cholesterol-rich liposomes during 10 h of incubation at 37 °C in a linear rate. Computer analysis for curve fitting of cholesterol uptake was performed with a programm-

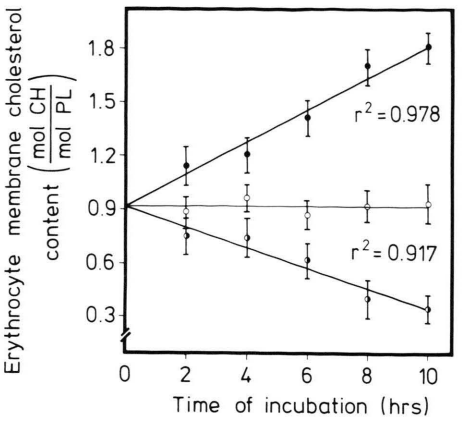

Fig. 1. Cholesterol enrichment and depletion of erythrocytes. RBCS (haematocrit 10%) were incubated in the absence of liposomes (○——○), in the presence of cholesterol/egg lecithin liposomes (2.5 mg liposomal cholesterol/ml) (●——●) or in the presence of egg lecithin liposomes (3 mg egg lecithin/ml) (◑——◑) in a medium consisting of 100 mM NaCl/15 mM KCl/10 mM dextrose/0.1 mM Na EDTA/8 mM Tris · HCl, pH 7.4 with 100 IU ml^{-1} penicillin-G and 100 μg ml^{-1} streptomycin sulphate. The incubations were performed with N_2 under gentle shaking in a water bath maintained at 37 °C. Points and bars represent means ± SD, respectively from three different experiments.

able Hewlett-Packard HP-97 calculator. The correlation coefficient (r^2) for linear fitting was greater than 0.95. The cholesterol content of erythrocyte membranes increased from the normal value CH/PL ~ 0.93 to a value of CH/PL ~ 1.8. The amount of cholesterol transfered to the membranes can be seen to vary not only with incubation time but also with concentration of cholesterol-rich liposomes. In parallel studies using cholesterol-free liposomes, the erythrocyte membrane cholesterol declined linearly to CH/PL molar ratio ~ 0.35 during 10 h of incubation. The incubation of cells in liposome free medium did not change the CH/PL molar ratio at all. It should be noted that the erythrocytes with a lower cholesterol content (CH/PL ratio < 0.3) were more fragile than the controls or cholesterol-enriched ones and frequently partially lysed before the ghosts were made. So in depletion experiments we tried not to exceed this value.

Our measurements show that the indigenous levels of cholesterol and phospholipids in rabbit RBC membranes are 0.270 ± 0.021 μmol/mg protein and 0.290 ± 0.020 μmol/mg protein, respectively. So the fraction incorporated into the membrane during 10 h incubation of RBCS with cholesterol-rich liposomes was 96% of the indigenous cholesterol and the fraction removed from the membrane during 10 h incubation of cells with egg lecithin liposomes was 65% of the indigenous cholesterol.

To link the structure-modifying effect of cholesterol incorporated within the RBC membranes to possible modulation of their function, the RBCS were incubated with cholesterol-rich liposomes and

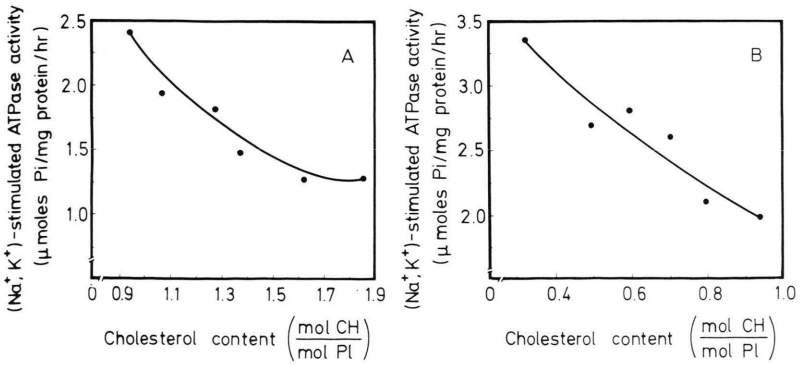

Fig. 2. A. Effect of cholesterol on (Na^+,K^+)-stimulated ATPase activity. Rabbit erythrocytes (haematocrit 10%) were incubated at 37 °C under N_2 in a shaking water bath with cholesterol/egg lecithin liposomes (2.5 mg liposomal cholesterol/ml). Aliquots of RBCS suspension were removed at 2 h intervals to determine CH/PL molar ratio and the activity of the enzyme in RBC membrane preparations. B. Effect of cholesterol depletion on the activation of (Na^+,K^+)-stimulated ATPase. Erythrocytes were incubated at 37 °C with egg lecithin liposomes (3 mg egg lecithin/ml). Each value represents the average of duplicate determinations of a typical experiment which has been repeated several times.

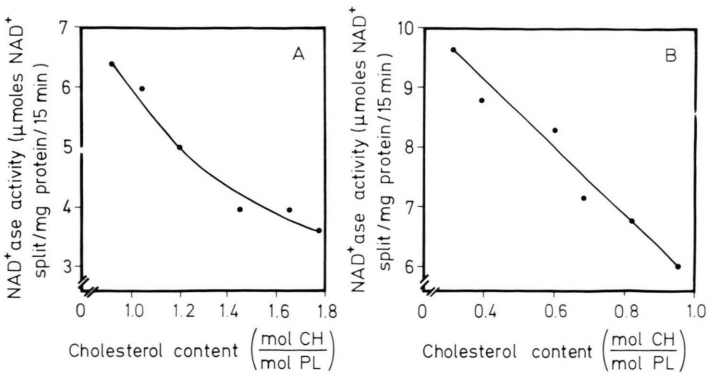

Fig. 3. NAD^+ase activity as a function of CH/PL molar ratio. A. Changes of NAD^+ase activity after incubation of erythrocytes with cholesterol/egg lecithin liposomes (2.5 mg liposomal cholesterol/ml). B. Changes of NAD^+ase activity after incubation of erythrocytes with egg lecithin liposomes (3 mg egg lecithin/ml). The procedure was the same as described in Fig. 2 A, B. The specific activity of the enzyme is expressed as μmoles NAD^+ split/mg membrane protein/15 min. Cholesterol content of the membranes is expressed as CH/PL molar ratio. Each value represents the average of duplicates from a typical experiment which has been repeated several times.

aliquots of cells were removed at 2 h intervals for assay of cholesterol, phospholipid phosphorus and enzyme activities in RBCS membrane preparations. Activities were expressed per mg of erythrocyte membrane proteins and plotted against cholesterol/phospholipid molar ratio. The effect of cholesterol on the activities of three membrane-bound enzymes (Na^+,K^+)-stimulated ATPase, NAD^+ase and acetylcholinesterase are shown in Fig. 2(A), 3(A) and 4. The activities of all enzymes decrease progressively at increasing cholesterol content of membranes over the range examined (2–10 h incubation with cholesterol/egg lecithin liposomes). (Na^+,K^+)-stimulated ATPase activity was decreased from 2.41 μmol Pi/mg protein/h for the control to 1.3 μmol Pi/mg protein/h for the cholesterol treated cells (54% of their control value). NAD^+ase activity was decreased from 6.4 μmol NAD^+ split/mg protein/15 min for the control to 3.6 μmol NAD^+ split/mg protein/15 min for the cholesterol treated cells (56% of their control value). Acetylcholinesterase activity was decreased from 0.383 ΔOD/mg protein/min for the control to 0.09 ΔOD/mg protein/min for cholesterol treated cells (23.5% of their control value). The present results confirm a high degree of sensitivity of the erythrocyte both vectorial and outer surface membrane enzymes to incorporated cholesterol. To eliminate the possibility that cholesterol interacts directly and irreversibly with RBC membrane-bound enzymes causing the loss of their activity, cholesterol-supplemented RBCS were incubated for 12 hours with egg lecithin liposomes which are known to remove cholesterol from biomembranes [12, 37].

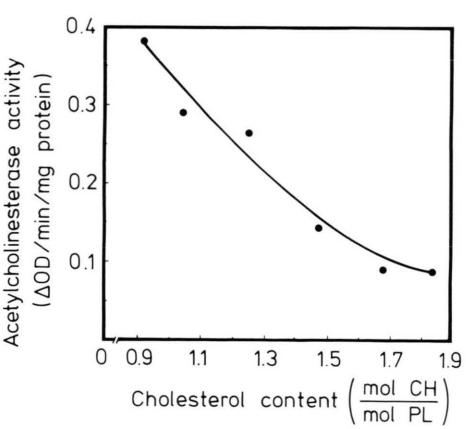

Fig. 4. Effect of cholesterol on acetylcholinesterase activity. Rabbit erythrocytes (haematocrit 10%) were incubated at 37 °C under N_2 in a shaking water bath with cholesterol/egg lecithin liposomes (2.5 mg cholesterol/ml). Aliquots of RBCS suspension were set aside at 2 h intervals to measure cholesterol and phospholipid concentration as well as the enzyme activity in RBCS ghost preparations. The indicated values represent average estimates of duplicate determinations of a typical experiment which has been repeated several times.

The results of re-extraction experiments are shown in Table. Although the change in CH/PL ratio provoked by incubation of erythrocytes with cholesterol-rich liposomes was totally reversible, the (Na^+,K^+)-stimulated ATPase and NAD^+ase activities were only partially restored (approx. to 80% of their initial levels), while the activity of acetylcholinesterase was restored completely. This indicates that cholesterol does not irreversibly change the erythrocyte membrane enzymes and its presence in the membrane causes mainly a modulation of the enzyme activities. Moreover, the removal of cholesterol from native RBC membrane results in a corresponding increase in (Na^+,K^+)-stimulated ATPase and NAD^+-

Table. Restoration of membrane-bound enzyme activities on removal of added cholesterol.

Enzymes tested	Untreated cells	Cholesterol/egg lecithin treated cells	Cholesterol/egg lecithin treated cells incubated with egg lecithin liposomes
	Cholesterol content (CH): 0.270 ± 0.021 μmol/mg protein Phospholipids content (PL): 0.290 ± 0.020 μmol/mg protein CH/PL molar ratio: 0.93	Cholesterol content (CH): 0.530 ± 0.036 μmol/mg protein Phospholipids content (PL): 0.294 ± 0.022 μmol/mg protein CH/PL molar ratio: 1.8	Cholesterol content (CH): 0.286 ± 0.025 μmol/mg protein Phospholipids content (PL): 0.298 ± 0.017 μmol/mg protein CH/PL molar ratio: 0.96
(Na^+,K^+)-stimulated ATPase	2.250 ± 0.161	1.358 ± 0.093*	1.804 ± 0.098**
NAD^+ase	6.20 ± 0.30	3.70 ± 0.025*	5.01 ± 0.027**
Acetylcholinesterase	0.360 ± 0.030	0.093 ± 0.011*	0.380 ± 0.038

RBCS (haematocrit 10%) were incubated at 37 °C for 10 hours with cholesterol/egg lecithin liposomes (2.5 mg liposomal cholesterol/ml). Cholesterol-treated cells were separated from residual cholesterol/egg lecithin vesicles and samples of RBCS suspension were taken for estimation of CH/PL molar ratio and specific enzyme activity in RBCS membrane preparations. Then washed RBCS were incubated with egg lecithin liposomes (3 mg phospholipid/ml) for 12 hours. The enzymes activities and CH/PL ratio were estimated in RBCS membrane preparations. (Na^+,K^+)-stimulated ATPase activity is expressed as μmol Pi/mg protein/h; NAD^+ase activity as μmol NAD^+ split/mg protein/15 min; Acetylcholinesterase activity as $\Delta OD_{412\ nm}$/mg protein/min. In each experiment duplicate samples were taken and the means of three separate experiments ± SD are given.

* Statistically significant compared to untreated erythrocytes ($p < 0.01$).
** Statistically significant compared to untreated and egg lecithin liposomes-treated erythrocytes ($p < 0.05$).

ase activities. It was observed that when CH/PL molar ratio falls from its normal value of approx. 0.93 to 0.31–0.35, the specific activity of (Na^+,K^+)-ATPase rises progressively from 2.1 μmol Pi/mg protein/h to 3.35 μmol Pi/mg protein/h (59% above of its original value) and the activity of NAD^+ase rises from 6.0 μmol NAD^+ split/mg protein/15 min to 9.63 μmol NAD^+ split/mg protein/15 min (60% above of its original value) (Figs. 2(B), 3(B)).

Discussion

When liposomes are used to alter the CH/PL molar ratio of erythrocyte membrane the question arises as to whether the alteration was a result of membrane-liposomes fusion, liposomes attachment to the membrane surface or a net exchange of cholesterol between the membrane and liposomes [15, 30, 31]. If the changes in CH/PL ratio are mainly due to the fusion of liposomes to the membrane or to their attachment to the membrane surface both cholesterol and phospholipid molecules of fused or attached liposomes would have to be added to the membrane. In such a case one can expect a significant increase in phospholipid/protein ratio of membranes obtained from liposome treated cells. Our results indicate that there was no statistically significant difference in phospholipid/protein ratio between liposome treated and control erythrocytes, which verifies a report that uncharged liposomes do not attach to erythrocytes [32]. The increase of membrane cholesterol can thus be regarded as being due to a selective uptake of cholesterol, perhaps from a water-soluble pool of cholesterol that had partitioned out of the vesicles [30].

In the present experiments the incubation of cholesterol-rich liposomes (CH/PL molar ratio approx. 2) with rabbit erythrocytes results in a net transfer of cholesterol from liposomes to erythrocytes which was linear with time over the 10 hours of the study. This procedure dublicates the cholesterol content of RBCS. Similarly, the rate of cholesterol depletion was linear with time and resulted in decrease of cholesterol content up to 40% of its initial level. The rates of cholesterol enrichment and depletion agree with those described by some authors, where the liposomes with high CH/PL ratio could increase the CH/PL molar ratio of RBC membranes up to 2–3 times [33] and are significantly higher than those described by others [34]. In this respect it is relevant to mention that significant changes in RBC cholesterol content can be seen in such pathological

conditions as severe liver disease, obstructive jaundice, Rh null disease and in experimentally-induced atherosclerosis [18, 35, 36].

Cooper *et al.* [37] have shown a direct relationship between CH/PL ratio and fluidity in red cell ghost membranes at physiological temperature, *i.e.* decrease in fluidity with increase in CH/PL ratio and vice versa. The decrease in the degrees of freedom of molecular movement in the hydrophobic core of RBC membrane produced by the addition of cholesterol to phospholipid bilayer, if felt by the proteins, would reduce the conformational flexibility of the membrane-bound enzymes. On the contrary, the decrease in cholesterol content would increase their conformational flexibility. If such flexibility is required for the activity of membrane-bound enzymes the cholesterol could have a controlling influence on the general metabolism of erythrocytes. The experimental results presented in this study indicate that cholesterol, when incorporated into RBC membrane, inhibits the activities of both vectorial and outer face oriented membrane-bound enzymes (Na^+,K^+)-stimulated ATPase and NAD^+ase-acetylcholinesterase respectively. This inhibition is proportional to CH/PL ratio in the membrane. The effect of cholesterol on enzyme activities was partially reversible in the case of (Na^+,K^+)-ATPase and NAD^+ase while the activity of acetylcholinesterase was completely restored after removal of cholesterol from cholesterol-enriched erythrocytes.

The fact that it was possible not only to reversibly inhibit the enzyme activities by cholesterol but also to increase their activities by cholesterol depletion procedures in an almost linear fashion, clearly indicates that the primary mechanism by which cholesterol modulates the enzyme activities is its reversible action on conformational flexibility of proteins through the alteration of membrane fluidity. The fact that the activities of two enzymes were not restored completely, while CH/PL ratio returned to its normal value, makes us to assume that cholesterol influences the enzyme activities in a more complex way than simply by modifying the membrane fluidity. Recently it was observed that cholesterol depletion of erythrocyte membrane causes a specific decrease in spectrin phosphorylation and an increase in lipid phosphorylation [38]. This finding was explaned by the action of cholesterol, perhaps through membrane fluidity, on the activity of membrane-bound proteases and kinases, enzymes involved in phosphorylation-dephosphorylation equilibrium of membrane proteins, which can influence their catalytic and structural functions. Apparently, such changes in membrane proteins could not be reversed by removal of cholesterol from the membrane. Concerning the erythrocyte membrane, conflicting views on the effect of cholesterol on the (Na^+,K^+)-ATPase transport function have been advanced. Owen and McIntyre [39] and Cooper *et al.* [19] suggested that the increased cholesterol content in erythrocytes of patients with liver diseases does not have a major effect on the activity of sodium pump. In contrast, Kroes and Ostwald [40] reported a 60% decrease in ouabain-sensitive sodium efflux from guinea pig erythrocytes enriched with cholesterol by dietary means. Similar observations were recently made by Yeagle [15] in the case of human erythrocytes. The inability in some cases to change the activity of transport protein by loading the intact membrane with cholesterol in combination with marked decrease of pump activity after addition of cholesterol to the phospholipid environment of isolated enzyme [41] led to the suggestion that cholesterol is excluded from the membrane areas containing the transport enzymes by a single shell of phospholipid bilayer (annulus) [42]. In the case of acetylcholinesterase, the fact that it is almost completely absent from both adults and children reticulocytes supports the hypothesis that the source of this enzyme is exogenous, perhaps the neuromuscular junctions [16]. Nevertheless, the finding that Lubrol-WX extraction did not abolish the break in the Arrhenius plot suggested that some phospholipid remains associated with solubilized enzyme. It was concluded that modulation of catalytic activity of acetylcholinesterase from bovine erythrocyte membrane results from fluidity changes in immobilized lipid, namely cardiolipin, rather than bulk lipids [43]. Similarly, Bloj *et al.* [44] could not find the correlation between the cholesterol content of erythrocyte membrane and the specific activity of acetylcholinesterase. The only interpretation possible for our results is that if the lipid annulus does exist around (Na^+,K^+)-ATPase and acetylcholinesterase then cholesterol is not excluded from it or the presence of this annulus does not protect these proteins from modulation by cholesterol, which could occur even at a distance. The last outer surface membrane enzyme, irrespective of its origin, is bound to the membrane strongly enough to be extremely sensitive to physico-chemical modification of erythro-

cyte membrane caused by cholesterol. In support of this notion is our recent observation that the decline of acetylcholinesterase activity towards the end of cell life could be partly determined by the interaction of this enzyme with membrane lipids during *in vivo* ageing of erythrocytes [12].

Since 20–50% of the total production of cellular ATP is used by the (Na^+,K^+)-ATPase [45] any effects of membrane cholesterol on ATP utilization by the (Na^+,K^+)-ATPase might have important consequences for cellular metabolism. For instance, one can expect that the increase in ATP content can lead to inhibition of glycolysis with concomitant decrease in 2,3 DPG and NADH production, substances necessary for physiological function of haemoglobin. If cation pumps, located in RBC membrane became inefficient due to the action of cholesterol the lack of osmotic regulation can lead to an influx of water into the cells with consequent disturbance in surface area/cell volume ratio and rapid destruction of erythrocytes. This may explain an occurrence of hemolytic anaemia in animals fed with cholesterol diet.

In conclusion, the activities of both vectorial and outer surface erythrocyte membrane-bound enzymes are a very sensitive indicator for interaction of cholesterol and erythrocyte membrane, which leads to changes in its physico-chemical properties. So it would be interesting to search for changes in cholesterol content and/or in membrane fluidity in a variety of pathological conditions associated with alteration in (Na^+,K^+)-ATPase or acetylcholinesterase activity in RBC membranes.

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