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STUDIES ON ($Na^+ + K^+$)-ACTIVATED ATPase

XLIX. CONTENT AND ROLE OF CHOLESTEROL AND OTHER NEUTRAL LIPIDS IN HIGHLY PURIFIED RABBIT KIDNEY ENZYME PREPARATION *

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(1) The neutral lipids and the free and bound fatty acids of a highly purified ($Na^+ + K^+$)-ATPase preparation from rabbit kidney outer medulla have been analysed. (2) On a dry weight basis, the total lipid content is nearly the same as the total protein content, and consists for 66% of phospholipids and for 34% of neutral lipids and free fatty acids. In the latter category cholesterol is the main component (71%). (3) On a molar basis the enzyme preparation contains 382 mol phospholipids, 67 mol free fatty acids, 9, 16 and 12 mol mono-, di- and triacylglycerols, 249 and 19 mol free and esterified cholesterol per mol enzyme. (4) The fatty acid composition of each lipid and of the free fatty acid fraction, present in the enzyme preparation, is reported. (5) All cholesterol and part of the phospholipids can be removed by hexane extraction, leaving 66% of the ($Na^+ + K^+$)-ATPase activity. Oxidation of all cholesterol to cholest-4-en-3-one by cholesterol oxidase leaves 85% of the ($Na^+ + K^+$)-ATPase activity. These results indicate that cholesterol is not essential for ($Na^+ + K^+$)-ATPase activity.

Introduction

Phospholipids have been most extensively studied in their relationship to the sodium pump enzyme system (see for review, Refs. 1 and 2). Cholesterol has so far received relatively little attention. The amount of cholesterol present in various ($Na^+ + K^+$)-ATPase preparations has been reported but without data on the degree of esterification [3–6]. The effect of cholesterol on ($Na^+ + K^+$)-ATPase activity is disputed, some authors claiming it to be essential for the activity [7–9], others denying its essential role [10,11]. None of these studies has been carried out on a highly purified ($Na^+ + K^+$)-ATPase preparation. No information has been published on the presence of neutral lipids and free and bound fatty acids in ($Na^+ + K^+$)-ATPase preparations.

In this study we present data on cholesterol and other neutral lipids and on free and bound fatty acids in a highly purified ($Na^+ + K^+$)-ATPase from rabbit kidney outer medulla. In particular, we report the results of studies on the effect of removal and modification of cholesterol on the enzyme activity.

Materials and Methods

Enzyme preparation

A highly purified membrane bound ($Na^+ + K^+$)-ATPase preparation from rabbit kidney outer medulla is obtained by the method of Jørgensen [3]. The specific activity ranges from 1.0 to 1.8 mmol ATP hydrolysed per mg protein per h. The enzyme preparation is stored at −20°C in 25 mM imidazole-HCl, 1 mM EDTA, 0.25 M sucrose (pH 7.5).

* For Part XLVIII see Ref. 14.
Abbreviation: BBOT, 2,5-bis[5′-*tert*-butylbenzoxazolyl-(2′)]-thiophene.

Protein determination

Protein is determined according to Lowry et al. [12] after trichloroacetic acid precipitation as described by Jørgensen [3]. Bovine serum albumin is used as a standard. In expressing lipid contents per mole enzyme we have used a protein molecular weight value of 326 800 [13] and multiplied the Lowry protein values by a factor 0.743 [14].

Total lipid extraction

Total extraction of lipids performed with chloroform/methanol (2 : 1, v/v), the combined lipid extracts are washed with 0.1 M KCl as described by Folch et al. [15].

Hexane extraction

The enzyme preparation is twice washed with distilled water and pelleted at 100 000 × *g* to remove buffer and sucrose. The pellet is dispersed in distilled water, the protein content is determined, and aliquots with known protein content are freeze-dried in polypropylene tubes (Greiner Labortechnik, F.R.G.) and stored at –20°C.

The dry enzyme preparation (150–300 μg protein) is extracted with 4 ml *n*-hexane (Merck, Darmstadt, F.R.G.; dried and stored over anhydrous Na_2SO_4) on a Griffin shaker for 5 min at room temperature. After centrifugation for 15 min at 40 000 × *g*, the supernatant is collected and the pellet is extracted once again in the same way. Both supernatant fractions are combined and evaporated in a stream of nitrogen.

Phospholipid analysis

Phospholipid analysis of the total lipid or hexane extract is carried out by two-dimensional thin-layer chromatography on silica gel, containing 4% alkaline magnesium silicate [16]. The phosphate content of the lipid spot is measured by a modified Fiske-Subba-Row method [16].

For fatty acid analysis, the phospholipids are visualized on the thin-layer plate by adding BBOT (10 mg/100 ml solvent; Sigma, St. Louis, MO, U.S.A.) to the second dimension eluting solvent system. The lipid spots are marked under ultraviolet light, and are scraped off. The material is extracted three times with 0.1 ml chloroform/methanol (2 : 1, v/v) without a KCl wash. The combined extracts are evaporated in a stream of nitrogen just before use.

Neutral lipid and free fatty acid analysis

Free fatty acids and neutral lipids are separated from the phospholipids by one-dimensional thin-layer chromatography on silicagel 60 plates (10 × 10 cm, Merck, Darmstadt, F.R.G.). The total lipid extract, containing maximally 3.3 μg lipid phosphorus, is applied in a line on the thin-layer plate. The plate is first eluted with chloroform and then with hexane/chloroform (3 : 1, v/v) [17]. The spots are made visible with water or iodine vapor, scraped off and extracted three times in 0.1 ml chloroform/methanol (2 : 1, v/v) without KCl wash.

Cholesterol determination

Total cholesterol is determined enzymatically by a modification of the CHOD-PAP method (Boehringer, Mannheim, F.R.G.). Total lipid extract or hexane extract of 100 μg ($Na^+ + K^+$)-ATPase protein is taken up in 25 μl isopropanol (Merck, Darmstadt, F.R.G.) and 1.0 ml cholesterol reagent is added. The mixture is incubated for 15 min at 37°C and the 500 nm absorbance read against a reagent blank. A standard curve of 0.4–2.0 mg/ml cholesterol in isopropanol is prepared with the 'Precimat' (Boehringer, Mannheim, F.R.G.) cholesterol standard solution.

Fatty acid analysis

Fatty acid analysis is carried out as described by Drenthe et al. [18] with two modifications: (1) tricosanoic acid (C23 : 0, Sigma, St. Louis, MO, U.S.A.) is used as internal standard, (2) the free fatty acids and neutral lipids, after separation by thin-layer chromatography, are extracted with chloroform/methanol as described above and methylation is carried out with 14% (w/v) boron trifluoride in methanol.

Glycerol determination

The lipid extract of a thin layer spot is taken up in 25 μl nonane (Fluka, F.R.G.). Glycerol is determined by the method of Giegel et al. [19] for triacylglycerols, with 20-fold reduction of all volumes. A standard curve of 0–0.5 mg/ml triolein (Sigma, St. Louis, MO, U.S.A.) is used.

Cholesterol oxidase incubation and cholest-4-en-3-one determination

To a series of tubes, each containing 75 μg (Na^+ +

K^+)-ATPase protein in 1 ml 50 mM Tris-HCl buffer (pH 7.4), 20 µl cholesterol esterase (7 units/ml) and 20 µl cholesterol oxidase (25 units/ml) (both obtained from Boehringer, Mannheim, F.R.G.) are added. The tubes are incubated at 37°C for various lengths of time, after which ($Na^+ + K^+$)-ATPase activity and cholestenon are determined along with untreated controls of ($Na^+ + K^+$)-ATPase.

Cholestenon is determined as described by Moore et al. [20], including the extraction with Dole reagent required for membrane preparations. Standard curves are prepared by adding 0–50 µg cholesterol in 25 µl isopropanol to 975 µl Tris/HCl buffer (pH 7.4), containing 0.1% deoxycholate (w/v) and 10 µl cholesterol oxidase (4 units/ml) and incubating for 3 h at 37°C.

Results

Lipid composition of the ($Na^+ + K^+$)-ATPase preparation

The free fatty acids and neutral lipids of the ($Na^+ + K^+$)-ATPase preparation are separated from the phospholipids by one-dimensional thin-layer chromatography. Two different solvent systems (see Ref. 17) are used to obtain effective separation of the various neutral lipids. (Fig. 1: track A shows the lipid pattern after spraying with water and track B after staining with iodine vapor). In the ($Na^+ + K^+$)-ATPase preparation we have quantitatively determined phospholipids, monoacylglycerols, free fatty acids, free cholesterol, esterified cholesterol, di- and tri-acylglycerols. These lipids are determined in extracts of the various spots by the following methods: glycerol determination (acylglycerols), total cholesterol determination (free and esterified cholesterol), phosphate determination (phospholipids) and fatty acid analysis (all lipids except free cholesterol). Some minor glycolipids may be present, which have not been analyzed.

The results of these quantitative determinations are combined in Table I. Two remarks are necessary for a proper understanding of the data in Table I. (1) The cholesterol ester content of the preparation is below the detection limit of the cholesterol determination method, but it could be determined by the more sensitive fatty acid analysis. (2) The free fatty acid content is calculated as the difference between

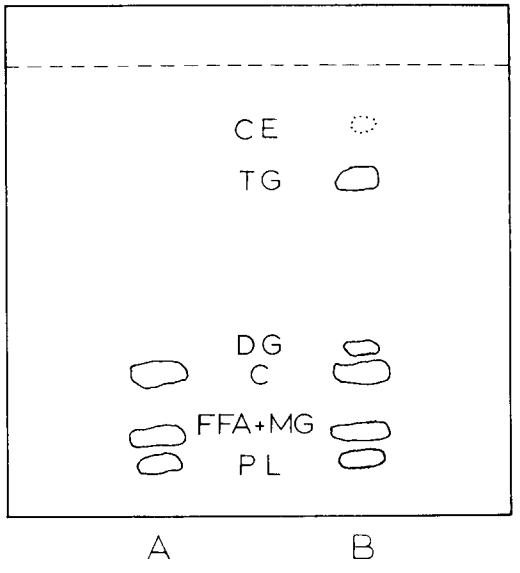

Fig. 1. Separation of free fatty acids, neutral lipids and phospholipids by one dimensional thin-layer chromatography. The technique is described under Methods. Track A shows the lipid pattern after visualization with water and Track B after staining with iodine vapor. Abbreviations: PL, phospholipids; FFA, free fatty acids; MG, monoacylglycerols; DG, diacylglycerols; TG, triacylglycerols; C, free cholesterol; CE, esterified cholesterol.

that in the combined free fatty-acid-monoacylglycerol spot on the thin-layer plate and the monoacylglycerol content obtained by the glycerol determination.

Table I shows that on a molar basis 51% of the total lipids analysed are phospholipids, 36% cholesterol, 5% acylglycerols (1.2% mono-, 2.1% di-, 1.6% triacylglycerols) and 9% free fatty acids.

Fatty acid composition of the lipids

The fatty acid analysis of the free fatty acids and neutral lipids is carried out after one-dimensional separation on thin layer chromatography. Since BBOT is insoluble in chloroform/hexane, ultraviolet spot detection by means of this scintillator is impossible. Therefore, two samples were run in parallel on one plate, and after separation one half of the plate is treated with iodine vapour for spot detection, so that the invisible spots on the other half can be located and scraped off for fatty acid analysis by gas-liquid chromatography.

TABLE I

LIPID COMPOSITION OF RABBIT KIDNEY ($Na^+ + K^+$)-ATPase PREPARATION

Values are given with standard error of the mean, the number of determinations is given in parentheses.

	Lipid content in μg lipid/mg protein, obtained through assay of:				Mol lipid/mol ($Na^+ + K^+$)-ATPase [c]	Percent of total lipids
	Glycerol	Cholesterol	Fatty acid	Phosphate		
Phospholipids				651 ± 42 (3)	382 ± 24 [d]	51 ± 3
Free fatty acids			} 48 ± 14 (3) [b]		67 ± 21 [e]	9 ± 3
Monoacylglycerols	7.3 ± 0.9 (4)				9 ± 1	1.2 ± 0.2
Diacylglycerols	23.5 ± 2.8 (2)		24.0 ± 1.8 (3)		16 ± 1	2.1 ± 0.2
Triacylglycerols	20.0 ± 3.6 (3)		25.4 ± 1.8 (4)		12 ± 1	1.6 ± 0.2
Free cholesterol		219 ± 6 (4)			249 ± 7	33 ± 1
Esterified cholesterol		n.d. [a]	24 ± 4		19 ± 3	2.5 ± 0.4
					754 (total)	

[a] n.d., not detectable.

[b] Monoacylglycerols and free fatty acids cannot be separated on this thin-layer chromatography system; the number given here is the weight of total fatty acids, belonging to monoacylglycerols and free fatty acids.

[c] Calculated from the previous columns by multiplication with a factor 1.345, to correct for the fasely high values of the Lowry protein determination [14,21] and by using a protein molecular weight value of 326 800 (ref. 13). Mean molecular weights are for: phospholipids, 750; free fatty acids, 277; monoacylglycerols, 351; diacylglycerols, 616; triacylglycerols, 877 and esterified cholesterol, 555.

[d] Consisting of sphingomyelin, phosphatidylcholine, phosphatidylserine, phosphatidylinositol and phosphatidylethanolamine: 68, 136, 50, 21 and 107 mol/mol enzyme, respectively (Ref. 2; see remark [c]).

[e] Calculated from [b] after correction for monoacylglycerols present.

Table II shows the fatty acid composition of the various lipids (phospholipids and neutral lipids) analysed in the ($Na^+ + K^+$)-ATPase preparation. It also gives the unsaturation index and average chain length (number of carbon atoms) of the fatty acids.

Cholesterol removal from the ($Na^+ + K^+$)-ATPase preparation by hexane extraction

After hexane extraction of the frozen-dried ($Na^+ + K^+$)-ATPase preparation the residual enzyme activity and the residual phospholipids are determined in the pellet, the phospholipids and cholesterol in the hexane extract. Table III shows that hexane extraction removes 100% of the cholesterol and 22–26% of the phospholipids. Under these circumstances only 34% of the ($Na^+ + K^+$)-ATPase activity is lost.

The composition of the extracted phospholipids does not differ greatly from that of the native enzyme as previously published by De Pont et al. [2]. This indicates that hexane does not preferentially extract one of the phospholipids.

Cholesterol oxidation

Incubation of the ($Na^+ + K^+$)-ATPase preparation with cholesterol esterase and cholesterol oxidase com-

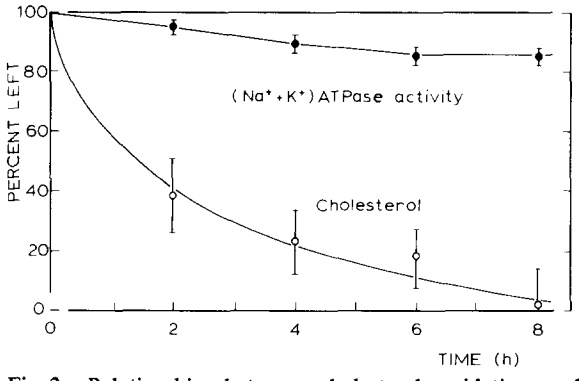

Fig. 2. Relationship between cholesterol oxidation and ($Na^+ + K^+$)-ATPase activity. The cholesterol oxidation is described under Methods. Percent residual cholesterol (○——○) and percent residual ($Na^+ + K^+$)-ATPase activity (●——●) are plotted against incubation time. Each point represents three measurements for each of three different preparations with the standard error of the mean.

TABLE II

FATTY ACID COMPOSITION OF LIPIDS IN ($Na^+ + K^+$)-ATPase PREPARATION

Composition is given in mol% with standard error of the mean for n determinations on n different preparations. Abbreviations: Sph, sphingomyelin; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; FFA, free fatty acid, MG, DG and TG, mono-, di- and triacylglycerol; CE, cholesterol ester.

Fatty acid	Sph (n = 3)	PC (n = 4)	PI (n = 4)	PS (n = 4)	PE (n = 4)	FFA + MG (n = 4)	DG (n = 3)	TG (n = 4)	CE (n = 3)
14 : 0	1.0 ± 0.6	2.9 ± 0.1	0.45 ± 0.05	1.1 ± 0.1	24 ± 1	2.6 ± 0.8	1.2 ± 0.6	10 ± 4	1.3 ± 1.3
16 : 0	31 ± 2	39 ± 2	16 ± 1	16 ± 1	9 ± 2	34 ± 1	29 ± 2	21 ± 0.2	30 ± 1
16 : 1	5 ± 2	2.7 ± 0.1	4 ± 1	2.5 ± 0.5	3 ± 1	3.8 ± 0.5	3.1 ± 0.2	5.5 ± 1	4 ± 2
18 : 0	13.2 ± 0.2	5.4 ± 0.1	27 ± 3	27 ± 1	6.0 ± 0.5	23 ± 1	18 ± 3	13 ± 2	17 ± 2
18 : 1	16 ± 1	29.3 ± 0.5	12 ± 1	27 ± 1	18 ± 1	19 ± 1	20.0 ± 0.6	13 ± 1.5	16 ± 1
18 : 2	13 ± 3	15.2 ± 0.2	11.8 ± 0.3	14.0 ± 0.5	9.0 ± 0.5	4.1 ± 0.8	7.2 ± 0.2	2.2 ± 0.2	
18 : 3		1.3 ± 0.1	0.3 ± 0.1	0.8 ± 0.1	1.0 ± 0.2	0.6 ± 0.2		1.3 ± 0.5	
20 : 1						0.9 ± 0.1	0.6 ± 0.3		
20 : 4	6 ± 3	2.7 ± 0.5	18 ± 1	9 ± 2	27 ± 1			10 ± 3	
20 : 5						0.5 ± 0.2		4 ± 2	
22 : 0	3.0 ± 0.6		0.9 ± 0.1		0.3 ± 0.1				
22 : 1						5.0 ± 0.3	3.6 ± 1.0		
22 : 5 ω6	13 ± 1	1.2 ± 0.1	9 ± 1	3.5 ± 0.2	1.6 ± 0.2	7 ± 3	17 ± 5	17 ± 5	31 ± 2
22 : 5 ω3								3.3 ± 1.4	
24 : 0					1.6 ± 0.4				
Average chain length [a]	18.2	17.1	18.2	18.0	17.6	17.1	17.65	17.5	17.35
Unsaturation index	136	83	158	113	158	76	124	188	175

[a] Value represents average number of carbon atoms in the fatty acids chains.

TABLE III

EFFECTS OF HEXANE EXTRACTION OF FROZEN-DRIED ($Na^+ + K^+$)-ATPase PREPARATION

Average values are given with the standard error of the mean for three determinations on three different preparations.

Pellet		Supernatant	
Rest activity (% of total)	Rest phospholipid (% of total)	Phospholipid extracted (% of total)	Cholesterol extracted (% of total)
66 ± 10	79 ± 6	26 ± 3	104 ± 3

pletely converts the cholesterol to cholest-4-en-3-one.

Fig. 2 shows that total oxidation of cholesterol is obtained after incubation for 8 h at 37°C. At that time the ($Na^+ + K^+$)-ATPase activity of a control preparation (no cholesterol esterase and oxidase added) has hardly decreased, while the treated preparation has lost only 15% activity relative to the control preparation.

Discussion

Lipid composition of the membrane

Since relatively little is known about the neutral lipids in membranes, we have compared our data for the rabbit kidney plasma membrane preparation with the few data reported for other membranes. The data in Table IV show that in all plasma membranes the major lipids are phospholipids and cholesterol (2 : 1 to 3 : 1 on weight basis) and that the cholesterol is almost exclusively non-esterified. All plasma membrane preparations contain considerable amounts of free fatty acids (except those from human erythrocytes) and also of acylglycerols. It is not clear whether these two lipid classes are present in the native membranes in such amounts, or that they are introduced during the isolation and purification steps.

Free fatty acids (67 mol/mol enzyme) are negatively charged at physiological pH, which means that they contribute significantly to the pool of negatively charged lipids besides the negatively charged phosphatidylserine and phosphatidylinositol (51 and 21 mol/mol enzyme, respectively). This fact has not yet been taken into account in the discussion about the role of negatively charged phospholipids (see Ref. 2) in the activity of ($Na^+ + K^+$)-ATPase.

Table V compares our values for the cholesterol and phospholipid contents with those published by others [3–6] for purified ($Na^+ + K^+$)-ATPase preparations. The rectal gland preparation is low in phospholipids, while the lamb kidney preparation is low in cholesterol. The molar cholesterol/phospholipid ratio generally approaches a value of one, indicating that

TABLE IV

LIPID COMPOSITION OF SOME PLASMA MEMBRANE PREPARATIONS

Lipid	Percent of dry weight [a] in membrane preparation				
	Rabbit kidney	Calf eye lens	Rat liver	Rat enterocyte	Human erythrocyte
Phospholipid	65.8	65	60	51.4	60
Cholesterol (free)	22.1	31	20	16	24
Cholesterol (esterified)	2.4		0.4	1.1	1.2
Free fatty acid	4.1	2.9	5.3	8.9	<0.2
Monoacylglycerol	0.7				} 1.5
Diacylglycerol	2.4				
Triacylglycerol	2.3	1.1	6.7	3.7	3
Glycolipid	n.d. [b]			19.3	10
Unknown			7.6		
Reference	This paper, Table I	22	23	24	25, 26

[a] Where necessary, these values are calculated from literature data, assuming the lipid molecular weights given in the legend of Table I.

[b] n.d., not determined.

TABLE V

TOTAL PHOSPHOLIPID AND CHOLESTEROL CONTENTS OF HIGHLY PURIFIED ($Na^+ + K^+$)-ATPASE PREPARATIONS FROM DIFFERENT SOURCES

Species	Ref.	Content (mg/mg protein)		Molar ratio [a] cholesterol/ phospholipid
		Phospholipid	Cholesterol	
Rabbit kidney	This paper	0.65	0.24	0.70
Rabbit kidney	3	0.73	0.30	0.81
Lamb kidney	5	0.825	0.12	0.27
Pig kidney	6	0.77	0.26	0.65
Rectal gland	4	0.39	>0.2	>1
Rectal gland (solubilized)	4	0.77	0.26	0.65

[a] Assuming an average molecular weight of 750 for the phospholipids; molecular weight of cholesterol is 387.

cholesterol is a major lipid component in these preparations. Only 7% of the cholesterol in our preparation is esterified (Table I), mostly with palmitic acid (C 16 : 0) and docosapentanoic acid (C 22 : 5; Table II).

Some minor glycolipids may be present in this kind of preparation, of which sulphatides may play a special role because in various tissues a linear relationship between ($Na^+ + K^+$)-ATPase activity and sulphatide content is found [27,28]. However, their presence is probably not essential for the enzyme activity [27].

Fatty acid composition

According to their fatty acid composition (Table II) the phospholipids can be divided into three classes: (1) the highly unsaturated phosphatidylinositol and phosphatidylethanolamine, the former distinguished by relatively long fatty acids, (2) the medium unsaturated phospholipids sphingomyelin and phosphatidylserine, (3) the highly saturated phosphatidylcholine, which carries relatively short fatty acids. Although there is no immediate explanation for the deviating fatty acid composition of phosphatidylcholine, it is striking that egg phosphatidylcholine, which is often used in phospholipid exchange experiments [29] and liposome/membrane interaction experiments [30], has nearly the same fatty acid composition (unsaturation index: 94 vs. 83; mean chain length: 17.4 vs. 17.1).

The fatty acid analysis of the free fatty acids and the neutral lipids (Table II) also indicates the existence of three classes: (1) highly unsaturated cholesterol esters and triacylglycerols, (2) medium unsaturated diacylglycerols, (3) highly saturated free fatty acids and monoacylglycerols. Since in the latter class only 10% of the fatty acids belong to monoacylglycerols and these have not been analyzed separately, we cannot conclude that the fatty acids of the monoacylglycerols are unsaturated. The most saturated lipids have the shortest acyl chain, as is also the case for phosphatidylcholine.

Cholesterol removal and modification

After lipid extraction, cholesterol was the most effective lipid in restoring ($Na^+ + K^+$)-ATPase activity in rat brain microsomes (Noguchi and Freed [7]), and in microsomes from *Electrophorus electric* organ (Järnefelt [8]). Hence, these investigators concluded that cholesterol would be essential for ($Na^+ + K^+$)-ATPase activity.

For erythrocyte ghosts Roelofsen et al. [10] and Zamudio et al. [31] and for rabbit kidney homogenate Wheeler and Isern de Caldentey [11] found that after removal of cholesterol by hexane or pentane extraction there was no large decrease in ($Na^+ + K^+$)-ATPase activity. We have, therefore, repeated the hexane extraction procedure on highly purified ($Na^+ + K^+$)-ATPase preparations from rabbit kidney. Table III shows that hexane extracts all cholesterol and 22–26% of the phospholipids. Fatty acid analysis of the hexane extract after separation on thin-layer

chromatography indicates that all free fatty acids and neutral lipids are also extracted. So only phospholipids and possibly some minor glycolipids are left in the membrane preparation. Since most of the $(Na^+ + K^+)$-ATPase activity (66%) is still present, we must conclude that cholesterol and other neutral lipids are not really essential for $(Na^+ + K^+)$-ATPase activity in this preparation.

Our results appear to contradict those of Claret et al. [32], who partly removed cholesterol from human erythrocyte membranes by incubation with phosphatidylcholine vesicles. This resulted in an increase in the maximal rate of Na^+ efflux and a lowering of the apparent affinity for intracellular Na^+. These changes in the kinetic parameters of the Na^+ efflux rate, however, were only found in the presence of a high intracellular K^+ concentration, but not with a low intracellular K^+ concentration. If cholesterol depletion would have the same effect on kidney membranes, the incubation medium for the $(Na^+ + K^+)$-ATPase assay (low K^+, high Na^+) would not be suitable for finding such an effect.

We have also tried to remove cholesterol by incubating the $(Na^+ + K^+)$-ATPase preparation with phosphatidylcholine vesicles, but we found no change in the cholesterol content. This contrasts with the finding for erythrocytes cited above and also with those for sarcoplasmic reticulum membranes [33], where cholesterol removal readily takes place in this way. Perhaps this requires a (protein) factor, which is absent from the purified kidney $(Na^+ + K^+)$-ATPase preparation.

In this context it is worth mentioning that we have not succeeded in replacing all phospholipids in the $(Na^+ + K^+)$-ATPase preparation by phosphatidylcholine by means of the method applied by Warren et al. [34] to $(Ca^{2+} + Mg^{2+})$-ATPase from sarcoplasmic reticulum. We achieved only partial replacement: up to 80% in preparations, where theoretically 97% phosphatidylcholine would be expected. This finding is in agreement with a recent rapport of Johannsson et al. [35], who report the same phenomenon for pig kidney $(Na^+ + K^+)$-ATPase. So in $(Na^+ + K^+)$-ATPase preparations, the lipids seem to be more tightly bound than in $(Ca^{2+} + Mg^{2+})$-ATPase preparations and part of them seems to be hardly exchangeable at all.

From studies of Bruckdorfer et al. [36], who replaced cholesterol in erythrocyte membranes by other steroids, including cholest-4-en-3-one, it is known that the hydroxyl group of cholesterol is very important for its association with other membrane constituents. However, complete conversion of the cholesterol in our purified $(Na^+ + K^+)$-ATPase preparation to cholest-4-en-3-one by a mild treatment with cholesterol esterase and cholesterol oxidase causes only 15% loss of $(Na^+ + K^+)$-ATPase activity (Fig. 2). The linear relationship between cholesterol oxidation and inhibition of $(Na^+ + K^+)$-ATPase activity, observed by Seiler and Fiehn [9] is probably due to secondary effects of cholesterol oxidation on the enzyme activity. Our results seem to justify the conclusion that cholesterol is not essential for $(Na^+ + K^+)$-ATPase activity.

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