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PROPERTIES OF $(Na^+ + K^+)$-ACTIVATED ATPase IN RAT LIVER PLASMA MEMBRANES ENRICHED WITH BILE CANALICULI

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SUMMARY

Liver plasma membranes enriched in bile canaliculi were isolated from rat liver by a modification of the technique of Song et al. (J. Cell Biol. (1969) 41, 124–132) in order to study the possible role of ATPase in bile secretion. Optimum conditions for assaying $(Na^+ + K^+)$-activated ATPase in this membrane fraction were defined using male rats averaging 220 g in weight. $(Na^+ + K^+)$-activated ATPase activity was documented by demonstrating specific cation requirements for Na^+ and K^+, while the divalent cation, Ca^{2+}, and the cardiac glycosides, ouabain and scillaren, were inhibitory. $(Na^+ + K^+)$-activated ATPase activity averaged 10.07 ± 2.80 μmol P_i/mg protein per h compared to 50.03 ± 11.41 for Mg^{2+}-activated ATPase and 58.66 ± 10.07 for 5′-nucleotidase. Concentrations of ouabain and scillaren which previously inhibited canalicular bile secretion in the isolated perfused rat liver produced complete inhibition of $(Na^+ + K^+)$-activated ATPase without any effect on Mg^{2+}-activated ATPase. Both $(Na^+ + K^+)$-activated ATPase and Mg^{2+}-activated ATPase demonstrated temperature dependence but differed in temperature optima. Temperature induced changes in specific activity of $(Na^+ + K^+)$-activated ATPase directly paralleled previously demonstrated temperature optima for bile secretion. These studies indicate that $(Na^+ + K^+)$-activated ATPase is present in fractions of rat liver plasma membranes that are highly enriched in bile canaliculi and provide a model for further study of the effects of various physiological and chemical modifiers of bile secretion and cholestasis.

INTRODUCTION

Bile is secreted from the hepatocyte by mechanisms which depend on the active transport of bile salts and a bile salt-independent pump which is poorly defined but present in a variety of animal species including man [1–9]. Bile salt-independent canalicular secretion accounts for 20–60 % of spontaneous bile flow depending on the species [7–9]. Previous evidence suggests that active sodium transport could account for this portion of bile secretion since cardiac glycosides, which inhibit sodium transport, were also found to inhibit bile flow in the rabbit [4] and isolated perfused rat liver [6].

If the hypothesis that sodium is actively transported from hepatocytes into bile is valid, then secretion should depend on the activity of $(Na^+ + K^+)$-activated ATPase, an enzyme which maintains water and electrolyte transport across most cell membranes, and regulates secretion across a number of specialized epithelial and glandular tissues, such as the intestine, renal tubule and salivary gland [10–13]. Isolation of membranes from tissues that transport sodium should result in purification of $(Na^+ + K^+)$-activated ATPase, and changes in the specific activity of this enzyme should correlate closely with alterations in secretion in vivo. In the present study membranes enriched in bile canaliculi were isolated from rat hepatocytes and optimum conditions for assaying $(Na^+ + K^+)$-activated ATPase were established. The properties of this enzyme were evaluated with reference to the possible role of $(Na^+ + K^+)$-activated ATPase as one determinant of bile salt-independent canalicular secretion in this species.

MATERIALS AND METHODS

Male albino Sprague-Dawley rats (Sprague-Dawley Co., Madison, Wisconsin, and Charles River Laboratories CD®, Wilmington, Massachusetts), weighing 175–275 g were used routinely for isolation of canalicular membranes while 100–150 g rats were obtained for studies of the effects of animal age on enzyme activity. All animals had free access to water, were fed Purina lab chow ad libitum, and were housed in a temperature-humidity-controlled room with 12 h light-dark cycles. Non-fasted animals were sacrificed between 8 and 10 a.m.

Membrane Isolation. Liver plasma membranes, rich in bile canaliculi were isolated by the discontinuous sucrose gradient method, essentially as described by Song et al. [14].Animals were decapitated at 5 °C. The livers were rapidly removed, placed on ice and perfused through the hepatic vein with ice-cold 1 mM $NaHCO_3$ buffer, pH 7.5, homogenized with loose fitting Dounce homogenizers (15 up and down strokes), then diluted with buffer, filtered through gauze to remove debris, and centrifuged at $1500 \times g$ for 10 min to collect the crude membrane fractions. Crude membranes were re-centrifuged in an angle rotor (Spinco 30) at $66\,000 \times g$ for 60 min (Beckman L2 ultracentrifuge) in a discontinuous sucrose gradient after gently mixing with density 1.26 (70.74%, w/v) sucrose. 14–16 ml of the sucrose/membrane mixture were layered over with 7 and 5ml sucrose solutions of density 1.18 (48.45 %, w/v) and 1.16 (42.97%, w/v), respectively. Canalicular membrane fractions were recovered form the density 1.16–1.18 interface and 4–5 vol of 1 mM $NaHCO_3$ buffer were added to dilute the sucrose. Pellets were obtained by centrifugation at 4 °C at $1500 \times g$ for 10 min in a Sorvall refrigerated centrifuge, using 50-ml tubes. Each pellet was washed a second time in 30–40 ml buffer and resuspended in distilled water to a final concentration of protein equalling 2–4 mg/ml. Membranes were then examined by $1000 \times$ phase microscopy for evidence of contamination from other cell fractions.

Protein was determined by the method of Lowry et al. [15] using Phenol reagent (Fisher Scientific). Crystallized bovine plasma albumin (Metrix-Armour Pharmaceutical Co.) was used as a standard. ATPase assays were performed on the day of preparation or after refrigeration at 5 °C overnight without loss of activity. Other enzyme assays were performed on both fresh and frozen material.

ATPase assays. Optimum assay conditions for determination of $(Na^+ + K^+)$-

activated ATPase in this membrane preparation are described in this report. The standard media for Mg^{2+}-activated ATPase (EC 3.6.1.4) included 150 mM NaCl and 20 mM imidazole buffer (Eastman Kodak, Inc.). The pH was adjusted to 7.8 at 37 °C [16]. Total ATPase was measured in identical media which also contained 5 mM KCl, and the difference in the two reactions represented $(Na^+ + K^+)$-activated ATPase. 50–150 μg of membrane protein, suspended in 50 μl of distilled water, was added to each reaction mixture and preincubated for 5–10 min in a Dubnoff shaker at 37 °C. 100 μl of a 0.125 M $MgCl_2$/disodium ATP (Sigma Chemical Co. A-3127) solution (final concentration of each equals 2.5 mM), were added to initiate the timed reaction which was carried out in a final volume of 5.0 ml. After exactly 15 min, 1 ml of ice-cold 35 % (w/v) trichloroacetic acid was added to terminate the reaction and the sample was placed immediately on ice, then centrifuged at 25 000 × *g* for 5–10 min to precipitate the protein. Aliquots of 1.5 ml were analyzed for inorganic phosphate by the Fiske-SubbaRow method [17]. Spontaneous hydrolysis of ATP was monitored by eliminating protein from control assays. Increases in spontaneous ATP hydrolysis were observed if the trichloroacetic acid supernatant was frozen overnight prior to phosphate determinations. All assays were performed in duplicate or triplicate.

Enzyme marker assays. Markers of subcellular fractions were monitored in representative membrane preparations and included 5′-nucleotidase (EC 3.1.3.5) (Song and Bodansky [18]) for plasma membranes, succinic acid dehydrogenase (EC 1.3.99.1) (Shephard and Hübscher [19]) for mitochondria, acid phosphatase (EC 3.1.3.2) (Appelmans et al. [20]) for lysosomes and glucose-6-phosphatase (EC 3.1.3.9) (Nordlie and Arion [21]) for microsomes.

Optimum conditions for assaying $(Na^+ + K^+)$-activated ATPase activity. These conditions were assessed by examining the effects of different concentrations of membrane protein, Na^+, K^+ and ATP in the reaction media. Temperature and pH optima were determined. The specificity of the $(Na^+ + K^+)$-activated ATPase activity was examined by studying the effects of varying concentrations of ouabain (Sigma Chemical Co.) and scillaren (Sandoz) as well as different cation and anion substitutions for Na^+, K^+ and Mg^{2+}.

Phase and electron microscopy. Although membrane preparations were examined routinely for purity by phase microscopy, representative preparations were pelleted, fixed at 5 °C for 2 h in 1 % O_sO_4 buffered with syncollidine at pH 7.4, embedded in Epon, thin sectioned and stained with uranyl acetate and lead citrate. Photomicrographs were obtained with a Siemens Elmiskop 1A electron microscope.

RESULTS

Plasma membrane fractions isolated from male Sprague-Dawley rats were uniformly enriched with plasma membranes consisting of canalicular bile fronts demarcated by tight junctions, as demonstrated by electron microscopy (Figs. 1 A and B). Microsomes and mitochondria were rarely identified, and enzyme marker studies revealed minimal contamination with non-plasma membrane material (Table 1). Phase microscopy characteristically demonstrated a uniform preparation of membrane fragments that were similar in appearance to those observed by electron microscopy (Fig. 2). Nuclei were rarely seen and these specimens were discarded. Protein yield from this isolation procedure averaged 0.32 mg protein/g rat liver and could be

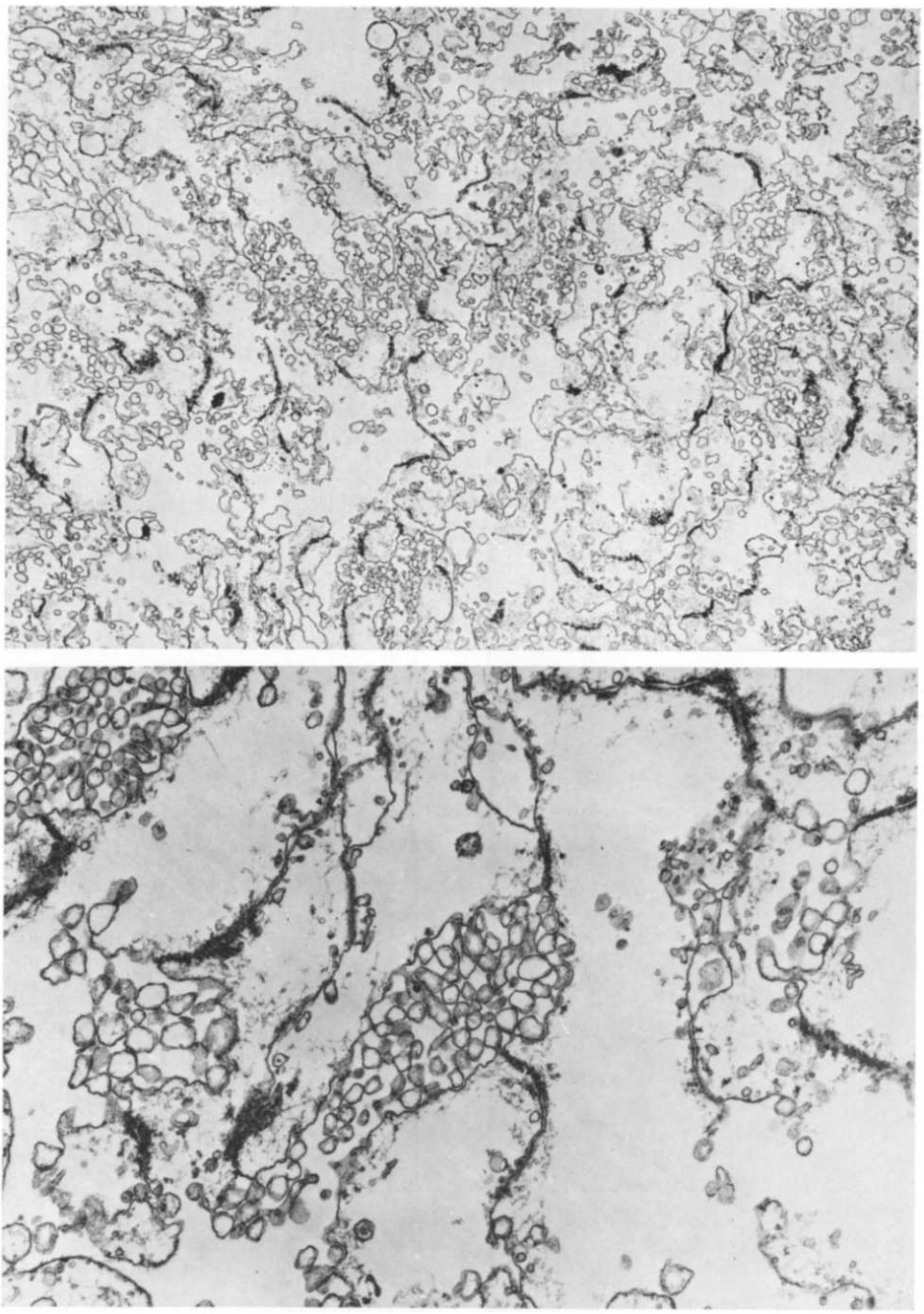

Fig. 1. (A) Electron micrograph of a representative field of rat liver plasma membranes, demonstrating the uniformity of the membrane preparation and enrichment with intact bile canaliculi. Canalicular structures can be easily identified by the double membrane-forming tight junctions (dark lines) which enclose vesicles that represent canalicular microvilli. (Original magnification · 2250.) (B) 7500 · original magnification of several bile canaliculi demonstrating numerous microvilli in cross section. A disrupted canaliculus is seen to the right.

TABLE I

ENZYME ACTIVITIES OF MARKERS OF SUBCELLULAR ORGANELLES AND PLASMA MEMBRANES

The number in parenthesis equals the number of assays performed on separate membrane preparations. The data listed are expressed as the mean ± S.D. Membranes were obtained from rats weighing 223 ± 46 g and liver that weighed 9.52 ± 1.02 g.

	Subcellular organelles		
	Glucose-6-phosphatase (μmol P_i/mg per h)	Acid phosphatase (μmol P_i/mg per h)	Succinate dehydrogenase (nmol formazan/mg per min)
Homogenate 162 ± 52 (mg protein/g liver) [18]	2.54 ± 0.93 (10)	1.19 ± 0.26 (10)	48.10 ± 16.40 (15)
Canalicular membranes 0.32 ± 0.11 (mg protein/g liver) [12]	0.98 ± 0.37 (7)	0.16 ± 0.38 (14)	3.17 ± 1.38 (6)
Membrane/homogenate ratio	0.38	0.13	0.06
	Plasma membrane enzymes (μmol P_i/mg per h)		
	5′-Nucleotidase	Mg^{2+}-activated ATPase	$(Na^+ + K^+)$-activated ATPase
Homogenate	3.07 ± 0.76 (10)	7.19 ± 0.72 (14)	0.45 ± 0.44 (14)
Canalicular membranes	58.66 ± 10.07 (24)	50.03 ± 11.41 (29)	10.07 ± 2.80 (29)
Membrane/homogenate ratio	19.1	6.96	22.38

increased by adding 0.5 mM Ca^{2+} to the 1 mM $NaHCO_3$ buffer as suggested by Ray [22] without significantly affecting marker enzyme activity or the electron microscopic appearance (Unpublished observations). However, in the present study, membrane isolation was performed without calcium additions.

Under optimal conditions defined in this study, membrane $(Na^+ + K^+)$-activated ATPase activity was reproducible and remained stable at 5 °C overnight. Freezing or subsequent storage usually resulted in diminished activity.

Fig. 3 illustrates a linear relationship between protein concentration and enzyme product over a 50–200 μg protein range. When Na^+ and K^+ concentrations were varied, activity increased with Na^+ concentrations up to 150 mM (Fig. 4), but diminished at 200 mM. Increases in K^+ concentrations above 5 mM were without effect. The choice of buffer (standardized at 37 °C) and pH were critical determinants of enzyme activity; imidazole was preferable to Tris for assay of $(Na^+ + K^+)$-activated ATPase and the optimum pH was 7.8. Mg^{2+}-activated ATPase was most active at pH 8.0 (Fig. 5). Optimal specific activities of $(Na^+ + K^+)$-activated ATPase and Mg^{2+} activated ATPase occurred with Mg^{2+} concentrations between 1.0 and 2.5 mM (Fig.

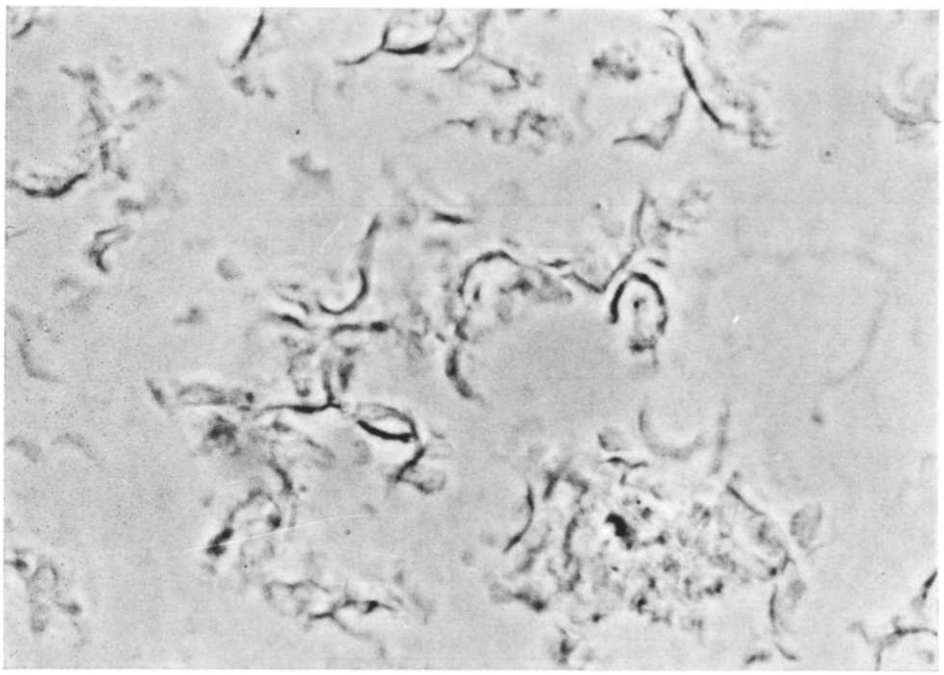

Fig. 2. 375 × magnification oil immerson phase micrograph of a typical membrane preparation, suspended in water. The dark lines represent highly refractile profiles of membranes seen in Figs 1A and 1B.

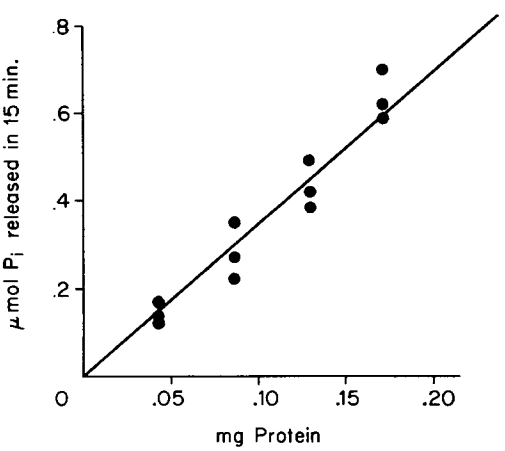

Fig. 3. Canalicular membrane ($Na^+ + K^+$)-activated ATPase activity as a function of protein concentration.

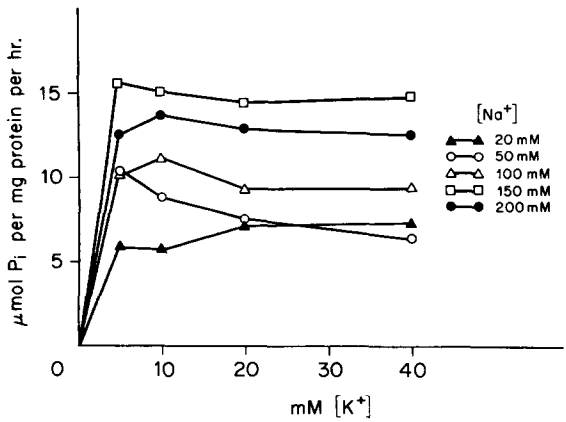

Fig. 4. The effect of varying the Na^+ and K^+ concentration on the specific activity of ($Na^+ + K^+$)-activated ATPase in canalicular plasma membranes. Each point represents the mean of duplicate assays. Optimum activity was observed with 5 mM K^+ and 150 mM Na^+. The reaction was carried out as 37 °C for 15 min in 20 mM imidazole buffer, pH 7.8, with 2.5 mM Mg^{2+}-ATP. Na^+ and K^+ (up to 50 mM) also promote a small stimulation in Mg^{2+}-activated ATPase activity as previously observed with enzyme preparations from other tissues and species [12].

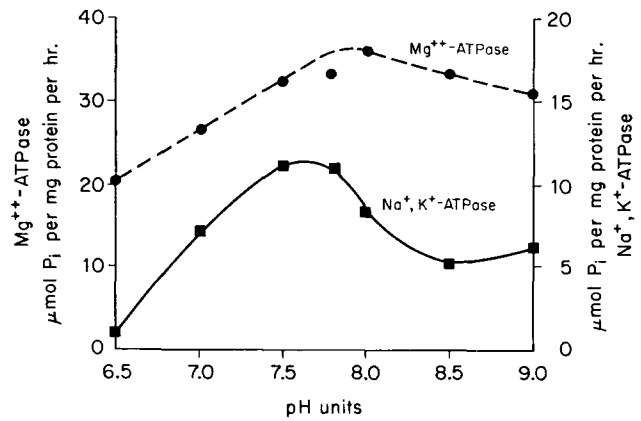

Fig. 5. Effect of pH on the specific activity of ($Na^+ + K^+$)-activated ATPase and Mg^{2+}-activated ATPase in a representative experiment. Imidazole buffer was used between a pH of 6.5–7.8 while Tris was used between 7.5 and 9.0. The pH was adjusted at 37 °C.

6). Higher Mg^{2+} concentrations resulted in diminished enzyme activity, particularly for Mg^{2+}-activated ATPase. Both ($Na^+ + K^+$)-activated ATPase and Mg^{2+}-activated ATPase activities were temperature dependent. Maximum ($Na^+ + K^+$)-activated ATPase was observed at 40–41 °C while Mg^{2+}-activated ATPase was optimal at 34–35 °C (Fig. 7). ($Na^+ + K^+$)-activated ATPase was progressively inhibited by increasing concentrations of either ouabain or scillaren, while neither cardiac glycoside had any effect on Mg^{2+}-activated ATPase. Complete inhibition of ($Na^+ + K^+$)-activated ATPase was observed at lower concentrations of scillaren than ouabain (Fig. 8). Both enzymes demonstrated a divalent cation requirement for Mg^{2+} while (Na^+

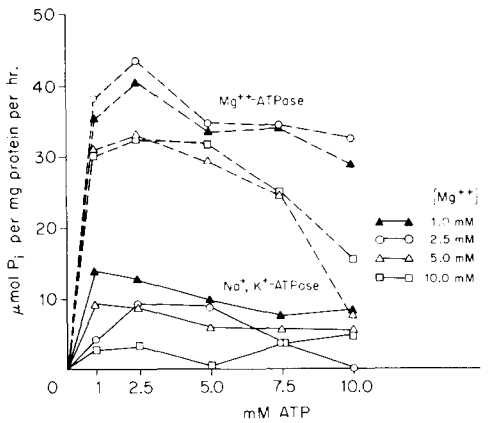

Fig. 6. Effect of varying concentrations of Mg^{2+} and ATP on the specific activity of $(Na^+ + K^+)$-activated ATPase and Mg^{2+}-activated ATPase in bile canalicular-enriched plasma membranes. Standard assay conditions were used for other reagents as described in Methods. Maximum activities were observed at an Mg^{2+}/ATP ratio of 1 : 1 (2.5 mM).

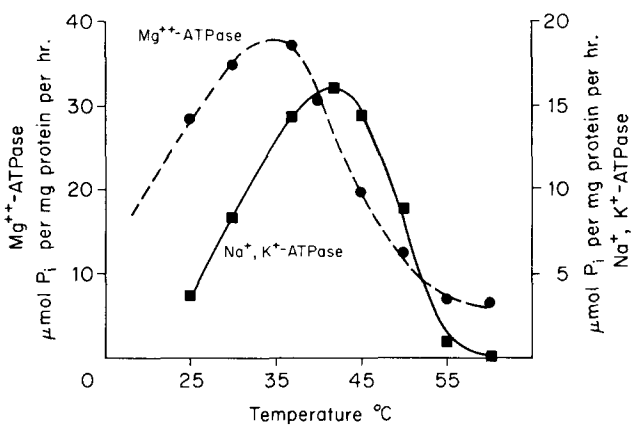

Fig. 7. Temperature dependence of Mg^{2+}-activated ATPase and $(Na^+ + K^+)$-activated ATPase specific activity in bile canalicular-enriched plasma membranes demonstrating dissociation of the temperature optima for the two enzymes used. Optimum activity of $(Na^+ + K^+)$-activated ATPase was observed at 40–42 °C while Mg^{2+}-activated ATPase activity was greatest at 32–34 °C.

$+K^+)$-activated ATPase was inactivated if Ca^{2+} (1.25 mM) was added to the reaction mixture or substituted for Mg^{2+} (2.5 mM). Despite the observation that Ca^{2+} inhibited $(Na^+ + K^+)$-activated ATPase, we did not observe an increase in $(Na^+ + K^+)$-activated ATPase if 1 mM EDTA was included in the standard assay. In contrast to the inhibitory effects of Ca^{2+} on $(Na^+ + K^+)$-activated ATPase, when Ca^{2+} was substituted for Mg^{2+}, the total ATPase activity was greater than with Mg^{2+} alone (Table II).

Table III illustrates the specificity of the monovalent cation requirements for $(Na^+ + K^+)$-activated ATPase in this membrane fraction. Li^+ did not substitute for

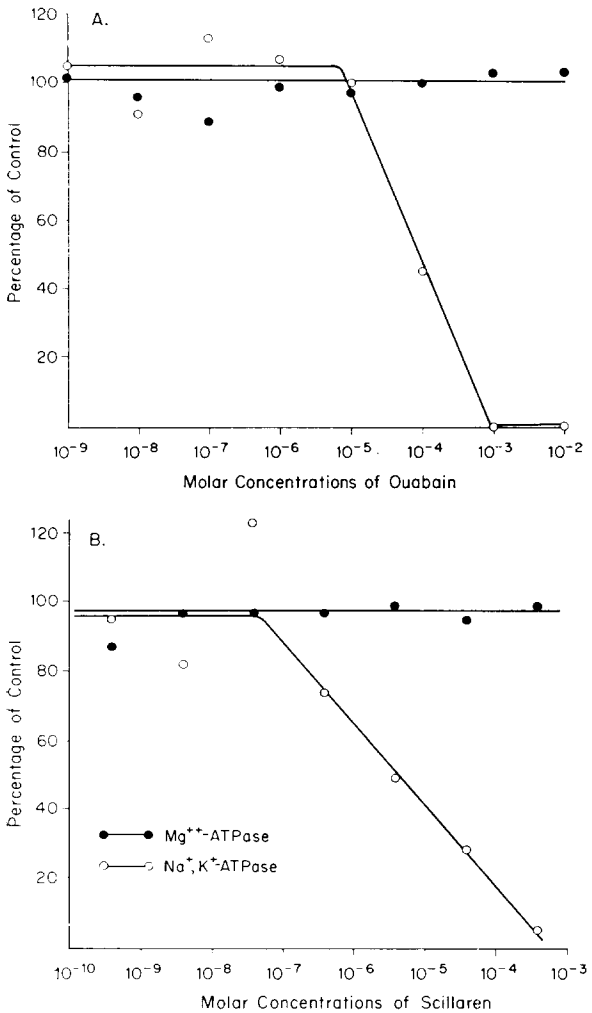

Fig. 8. The effect of increasing concentrations of ouabain (A) and scillaren (B) on $(Na^+ + K^+)$-activated ATPase and Mg^{2+}-activated ATPase activity in bile canalicular-enriched plasma membranes. Neither glycoside affected Mg^{2+}-activated ATPase while $(Na^+ + K^+)$-activated ATPase was completely inhibited at 10^{-3} M ouabain and $5 \cdot 10^{-4}$ M scillaren. Specific activity is expressed as percent of control values. Standard assay conditions were used.

Na^+, while Rb^+ restored activity to 44 % of control values. Neither Li^+ nor choline would substitute for K^+ while Rb^+ nearly restored activity to control values. In contrast, neither Na^+ nor K^+ was required for Mg^{2+}-activated ATPase although small increments of Mg^{2+}-activated ATPase were seen with addition of Na^+ or substitution with Rb^+ as previously reported [12, 23] (Table III). There was no specific anion requirement since SO_4^{2-}, Br^-, I^- and isethionate were without significant effect on either $(Na^+ + K^+)$-activated ATPase or Mg^{2+}-activated ATPase when substituted for Cl^- in the assay (Table IV).

The effect of animal age on ATPase activity is seen in Fig. 9. $(Na^+ + K^+)$-acti-

TABLE II

EFFECT OF DIVALENT CATIONS (Mg^{2+} AND Ca^{2+}) ON ATPase IN BILE CANALICULAR MEMBRANES

The values (expressed as percent of control values), represent the mean and standard deviation of six assays obtained from two membrane preparations. Ca^{2+} inhibited ($Na^+ + K^+$)-activated ATPase but significantly stimulated ATPase in the presence ($P < 0.01$) or absence of Mg^{2+} ($P < 0.001$). The assay medium contained 20 mM imidazole buffer, 150 mM Na^+, 2.5 mM ATP and the designated divalent cation.

Divalent cations	Percent of control values	
	($Na^+ + K^+$)-activated ATPase (%)	Mg^{2+}-activated ATPase (%)
2.5 mM Mg^{2+}	100 (control)	100 (control)
No Mg^{2+} or Ca^{2+}	7.1±4.0	10.7±2.0
Mg^{2+} (1.25 mM) and Ca^{2+} (1.25 mM)	9.15±12.5	111.1±4.3
Ca^{2+} only (2.5 mM)	9.4±16.3	127.8±5.8

TABLE III

MONOVALENT CATION REQUIREMENT FOR ATPase IN BILE CANALICULAR MEMBRANES

The values (expressed as percent of controls) represent the mean and standard deviation of six experiments obtained from two membrane preparations. Standard assay conditions were used except that Tris · ATP was substituted for disodium ATP. Mg^{2+}-activated ATPase values were obtained in the presence of Na^+ or Na^+ substitutes, while ($Na^+ + K^+$)-activated ATPase activity represents the increase in ATPase activity occurring after addition of K^+ or K^+ substitutes.

Monovalent cations		Percent of control values	
150 mM	5 mM	($Na^+ + K^+$)-activated ATPase (%)	Mg^{2+}-activated ATPase (%)
Na^+	—	—	100 (control)
Na^+	K^+	100 (control)	—
Li^+	—	—	89.6±5.5
Li^+	K^+	5.3±7.5	—
Rb^+	—	—	98.5±3.9
Rb^+	K^+	44.2±17.4	—
Na^+	Li^+	0	—
Na^+	Choline	2.2±3.3	—
Na^+	Rb^+	80.7±40.8	—

vated ATPase activity remained relatively constant until animals exceeded 8 weeks of age (250–260 g in body weight) when slightly lower values were observed. At the same time, Mg^{2+}-activated ATPase values increased substantially, reaching values by 11 weeks that were 170 % of values obtained in younger rats averaging 220 g in body weight.

TABLE IV

EFFECT OF Cl^- REPLACEMENT ON BILE CANALICULAR MEMBRANE ATPases

The pH was adjusted with H_2SO_4 and $MgCl_2$ was replaced with $MgSO_4$. The values are expressed as percent of control values when Cl^- was the major anion and are represented as the mean and standard deviation of three separate assays.

Anion	$(Na^+ + K^+)$-activated ATPase (%)	Mg^{2+}-activated ATPase (%)
Cl^-	100 (control)	100 (control)
SO_4^-	119.3±6.2	92.4±1.7
Br^-	117.8±10.4	93.9±2.8
I^-	96.0±13.1	97.0±3.6
Isethionate	83.0±7.8	101.1±2.1

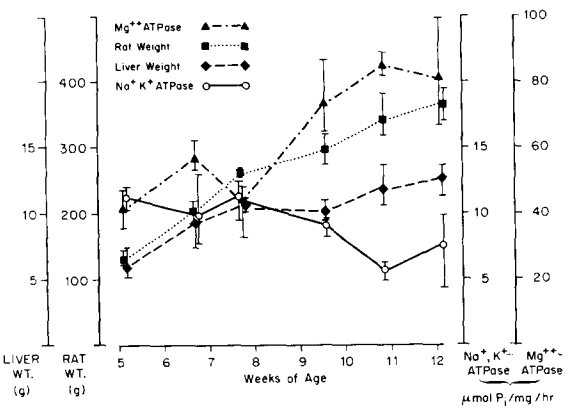

Fig. 9. The effects of age and weight of male Sprague-Dawley rats on the specific activity of $(Na^+ + K^+)$-activated ATPase and Mg^{2+}-activated ATPase in bile canalicular membranes. All animals were maintained on lab chow ad libitum. Membranes were prepared and assayed separately for each animal and three animals were sacrificed at each time point. Values are expressed as the mean and standard deviation. Substantial increments in the activity of Mg^{2+}-activated ATPase were observed at 8 weeks of age while $(Na^+ + K^+)$-activated ATPase activity diminished.

DISCUSSION

The results of the present study have characterized $(Na^+ + K^+)$-activated ATPase activity in a plasma membrane fraction that is enriched in bile canaliculi. While the precise location of the enzyme has not been established, the technique for isolation of plasma membranes used in this study resulted in a fraction of rat liver plasma membrane, which was highly enriched in canalicular structures as originally observed by Song et al. [14]. We have confirmed their findings in the present study, since bile canaliculi and tight junctions were seen throughout the electron microscopic field and these structures usually maintained the morphological characteristics of canaliculi observed in the intact cell [24]. Undoubtedly, fragments of plasma membranes from sinusoidal surfaces of the cell contribute to the total membrane popula-

lation although, on the basis of electron microscopic examination, the contribution of these fragments would seem to be small. According to present concepts [25], the homogenization procedure will fragment the sinusoidal and lateral cell wall membranes and form small vesicles, while canalicular membranes remain relatively protected from disruption because of the numerous terminal bars and tight junctions. The more fragmented plasma membrane vesicles then tend to enter the mitochondrial and microsomal fractions during the isolation procedure. It also seems unlikely that significant amounts of $(Na^+ + K^+)$-activated ATPase were present in other subcellular particles in our preparation, since plasma membrane enzyme markers were selectively purified while the specific activities of other subcellular organelles were diminished.

The present technique for isolating bile canalicular enriched plasma membranes differs from the procedure of Song et al. [14] in that male rather than female rats were used and the liver was perfused prior to homogenization in order to remove blood elements. Furthermore, membrane yields were doubled by gently stirring the membranes in sucrose prior to ultracentrifugation rather than mixing membranes and sucrose in a Dounce homogenizer. Specific activity of plasma membrane markers (Mg^{2+}-activated ATPase and 5'-nucleotidase) were generally comparable to values obtained in other rat liver plasma membrane preparations [22, 26–31], although intact canalicular structures were more plentiful. Therefore, as suggested by Song et al. [14], these bile canalicular plasma membrane fractions appear to be distinguished from other plasma membrane preparations primarily by the morphologic evidence that these fractions are highly enriched with bile canalicular structures. Contamination with other subcellular particles was always minimal, as judged by both electron and phase microscopy, since mitochondria or nuclei were rarely observed. Relatively small amounts of microsomal contamination were present as reflected by glucose-6-phosphatase activities and values were lower than in previous reports where female rats were used [14]. Lysosomal and mitochondrial enzymes (acid phosphatase, EC 3.1.3.2 and succinic acid dehydrogenase, EC 1.3.99.1) were not assayed by Song et al. [14] in this preparation and the present studies suggest that contamination with these subcellular organelles was also minimal.

$(Na^+ + K^+)$-activated ATPase has been studied extensively in rat liver homogenate by Bakkeren and Bonting [23, 32] but only Emmelot and Bos [27, 28, 33] have studied the properties of this enzyme in rat liver plasma membrane in detail. These authors utilized plasma membrane prepared by the Neville procedure [26], which may contain larger amounts of microsomal protein as determined by glucose-6-phosphatase activity.

Certain differences exist in the optimum conditions for assaying $(Na^+ + K^+)$-activated ATPase in the present study when compared to the Emmelot and Bos procedure [27]. Sodium concentrations were 150 mM rather than 66 mM, and a Mg^{2+}-activated ATPase ratio of 1:1 was preferable to 1:2. Imidazole buffer (20 mM) was found to be better than Tris and the pH optimum was 7.8 at 37 °C, rather than at 7.4 as previously reported [28].

Although $(Na^+ + K^+)$-activated ATPase is localized on cell membranes in many different tissues where the enzyme regulates the transport of Na^+ and K^+ [10–13], it has often been difficult to measure in preparations of rat liver plasma membranes, and was inconsistently present in low activity in canalicular membrane preparations examined by Song et al. [14]. In rat liver, $(Na^+ + K^+)$-activated ATPase represents a

small percentage of total ATPase activity [12, 23] (averaging 17% in the present study), a problem which may have accounted for difficulty in demonstrating ($Na^+ + K^+$)-activated ATPase consistently in some previous reports [14]. The effect of animal age may also be a factor since ($Na^+ + K^+$)-activated ATPase represents a smaller percentage of total ATPase activity in older animals (Fig. 9). In the present study ($Na^+ + K^+$)-activated ATPase activity was consistently recovered from canalicular-enriched plasma membranes once optimum conditions for its assay were defined. The presence of ($Na^+ + K^+$)-activated ATPase in canalicular-enriched plasma membranes was established by demonstrating a specific monovalent cation requirement for both Na^+ and K^+; by inhibiting activity completely with the cardiac glycosides, ouabain and scillaren, as well as the divalent cation Ca^{2+} [34]; and by increasing specific activity when Na^+ concentrations were increased in the media. These properties of ($Na^+ + K^+$)-activated ATPase are characteristic of this enzyme in a variety of tissues [10–13].

Although the present studies do not establish that this enzyme is localized to the canaliculus per se, or indicate that ($Na^+ + K^+$)-activated ATPase is important in the secretion of bile salt-independent flow, the properties of ($Na^+ + K^+$)-activated ATPase in this membrane preparation are consistent with this concept and provide a rationale for further study of this problem. For example, although Mg^{2+}-activated ATPase activity was maximum at 35 °C, which was identical to the findings of Emmelot and Bos [33], ($Na^+ + K^+$)-activated ATPase activity reached optimum activity at 40–41 °C (Fig. 7), declining thereafter to minimum levels at 50 °C. In the studies of Emmelot and Bos [33], the specific activity of ($Na^+ + K^+$)-activated ATPase increased between 40 and 50 °C by a factor of 2. This descrepancy in temperature optimum for ($Na^+ + K^+$)-activated ATPase suggests that there are important differences in ($Na^+ + K^+$)-activated ATPase as isolated and assayed by the two different techniques. Whether alterations in temperature optima relate to compositional changes in the membranes brought about by the differences in isolation techniques, or to differences in the enrichment of bile canaliculi remains speculative. Nevertheless, it is of interest that in the isolated perfused rat liver, bile secretion is essentially independent of bile salt excretion [5, 6] and demonstrates a temperature dependence which exactly parallels the stimulation and inhibition of ($Na^+ + K^+$)-activated ATPase observed in vitro in the present studies [35].

Since canalicular membrane Mg^{2+}-activated ATPase was relatively unaffected by ouabain and scillaren, and Mg^{2+}-activated ATPase temperature curves did not parallel changes in bile secretion, Mg^{2+}-activated ATPase may not play a predominant role in bile formation even though its activity is reduced by various experimental or physiologic conditions associated with reductions in bile flow [36–38]. However, much further study of these membrane enzymes will be necessary before more precise functional relationships can be established.

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