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CONTRAST MANIFESTATION OF ALKALINE PHOSPHATASE AND 5′-NUCLEOTIDASE IN PLASMA MEMBRANES ISOLATED FROM RAT LIVER AND ASCITES HEPATOMA

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Summary

1. Plasma membranes were isolated from ascites hepatoma AH-130 and rat livers with or without partial hepatectomy or bile duct ligation. Reciprocal manifestations of two marker enzymes for plasma membranes were observed in these membrane preparations; alkaline phosphatase activity was found much higher in the hepatoma membrane than in any preparations of the liver membranes, whereas 5′-nucleotidase activity was much lower in the former than in the latter.

2. Effects of lectins and anti-plasma membrane antiserum on these two marker enzymes were examined. The results showed that about 50% of apparent activity of 5′-nucleotidase found in the hepatoma membrane was exhibited by alkaline phosphatase.

3. Localizations of alkaline phosphatase and 5′-nucleotidase in polyacrylamide gels after electrophoresis were demonstrated using 5′-AMP and 5-Br, 4-Cl-indoxylphosphate as substrate. There was a difference in electrophoretic mobility between the alkaline phosphatase of the hepatoma and that of the liver.

Introduction

It has been suggested from a number of different lines of evidence that the cell surface or plasma membrane is involved in the regulation of cell growth [1]. Changes in the plasma mmebranes as a function of malignant transformation were demonstrated by various methods, such as cell agglutination by lectins [2], chemical analyses of glycoproteins [3] and glycolipids [4] and sodium dodecyl sulfate gel electrophoresis of membrane proteins [5]. Alterations in the structure and composition of the plasma membranes are likely to

affect the behavior of membrane-bound enzymes. Potential usefulness of membrane-bound enzymes in monitoring cell membrane changes during neoplastic transformation is of considerable interest, but has yet to be clearly demonstrated. Presence at cell surfaces of enzymes involved in nucleotide metabolism has been well established [6—8], although what roles these enzymes play in general or for individual cell types remain obscure.

In the present paper we describe a contrast manifestation of two marker enzymes for plasma membranes between the rat liver and the transplantable ascites hepatoma AH-130; the great increase of alkaline phosphatase activity and decrease of 5′-nucleotidase activity in the hepatoma membranes compared to those in the liver membranes.

Materials and Methods

Animals and tumor cells. Male rats, Donryu strain, were obtained from the Animal Supply Center of Kyuhsu University. Ascties hepatoma AH-130 cells [9] were harvested from the intraperitoneal cavity of Donryu rats which had been inocculated 7—9 days earlier.

Isolation of plasma membranes from rat liver and ascites hepatoma. Plasma membranes were isolated from rat liver and hepatoma by a slight modification of the original methods of Ray [10] and Emmelot and Bos [11], respectively.

Preparation of lectins. Ricinus communis agglutinin was isolated from *Ricinus communis* seeds and purified according to the methods of Tomita et al. [12] and Nicolson and Blaustein [13]. Wheat germ agglutinin was purified from wheat germ lipase (Sigma Chemicals, St. Louis, U.S.A.) according to the procedure described by Nagata and Burger [14]. The purified lectins were found to comprise a single protein in polyacrylamide gel electrophoresis. Concanavalin A was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden.

Preparation of anti-plasma membrane antisera. Antisera against the plasma membranes isolated from rat liver and hepatoma were separately prepared in rabbits by the process described previously [15].

Enzyme assays. Unless otherwise indicated, 5′-nucleotidase activity was determined in 0.5 ml of a reaction mixture containing 0.1 M Tris · HCl (pH 7.5), 5 mM $MgCl_2$, 0.1 M KCl, and 5 mM 5′-AMP. Incubations were carried out at 37°C for 20 min and terminated by the addition of 0.5 ml of 10% trichloroacetic acid. After removal of protein by centrifugation, P_i in the supernatant was determined according to Fiske and SubbaRow [16]. Control incubations contained no enzyme preparations, which were added after the addition of trichloroacetic acid. 5′-AMPase activity referred to activity of enzyme(s) which hydrolyzed 5′-AMP, irrespective of any pH value of the reaction medium used. Alkaline phosphatase activity was determined using *p*-nitrophenyl phosphate as substrate [17]. Assay mixture (1.0 ml) contained 0.2 M Ammediol (2-amino-2-methyl-1,3-propandiol) · HCl, pH 10.4, 5 mM $MgCl_2$, 10 mM *p*-nitrophenylphosphate. The formation of *p*-nitrophenol during 10 min incubation at 37°C was estimated spectrophotometrically at 400 nm. A unit of enzyme was defined as the amount that hydrolyzed one nmol of substrate per min.

Solubilization of enzymes from plasma membranes. Plasma membranes were suspended to about 10 mg protein/ml in 10 mM Tris · HCl buffer (pH 7.5) con-

taining 1 mM $MgCl_2$, treated with *n*-butanol [18] and centrifuged at 20 000 × *g* for 20 min. Triton X-100 was added to the aqueous phase obtained to 0.5%, and the solution was dialyzed against 10 mM Tris · HCl buffer (pH 7.5) containing 0.2 mM $MgCl_2$. The dialyzed samples were then centrifuged at 105 000 × *g* for 30 min and the supernatants were used as enzyme preparations for gel electrophoresis.

Standard polyacrylamide gel electrophoresis. Polyacrylamide gels were prepared in glass tubes (size; 5 × 100 mm) according to Davis [19], except that Triton X-100 and $MgCl_2$ were added to a final concentration of 0.1% and 0.2 mM, respectively, and that samples were applied directly to the separating gels after sucrose was added to the samples to 5%. Running buffer (50 mM Tris · glycine, pH 8.9) in electrophoresis also contained 0.1% Triton X-100 and 0.2 mM $MgCl_2$. Electrophoresis was carried out at the constant current of 3 mA/tube for 3 h at 2—4°C.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis. Preparation of gels and electrophoresis were carried out according to the method of Fairbanks et al. [20].

Samples in 0.2 M phosphate buffer, pH 7.5, containing 1% sodium dodecyl sulfate and 10% sucrose were incubated at 37°C for 30 min, and aliquots (20—50 units of activity) were loaded on each gel. After electrophoresis at 5 mA/gel for 3 h at room temperature, gels were stained for location of alkaline phosphatase as described later, or stained for protein [20]. Standard proteins for molecular weight determination, rat serum albumin and anti-rat serum albumin IgG, were prepared as described previously [21,22]. Immuno-complex between albumin and anti-albumin IgG was solubilized by incubation at 37°C for 30 min in 0.2 M phosphate buffer (pH 7.5) containing 1% sodium dodecyl sulfate and 1% β-mercaptoethanol, which gave albumin (68 000), and heavy chain (55 000) and light chain (23 000) of IgG. In the absence of β-mercaptoethanol, the same treatment of the immuno-complex gave a native IgG (160 000) and albumin.

Demonstration of enzymes in polyacrylamide gels. Localization of 5′-AMPase in the gels was achieved by a modification of the method developed for alkaline phosphatase [23]. A substrate solution contained the following final concentrations of reagents; 10 mM 5′-AMP, 23 mM $CaCl_2$, 5 mM $MgCl_2$, and 0.5 M Tris · HCl (pH 7.5). Demonstration of alkaline phosphatase was carried out using 5-Br, 4-Cl-indoxylphosphate as substrate. A substrate solution contained the following final concentrations of reagents; 1 mM 5-Br, 4-Cl-indoxylphosphate, 5 mM $MgCl_2$, and 0.48 M Ammediol · HCl buffer (pH 10.4). The gels removed from the columns were immediately incubated at 37°C in the above substrate solutions until the white band could be seen in the gels for 5′-AMPase and a green band for alkaline phosphatase (usually 15—30 min).

Materials. Sephadex G-100 and Sepharose 4B were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden, and 5′-AMP from Boehringer GmbH., Mannheim, Germany. 5-Br, 4-Cl-indoxylphosphate was generously provided by Dr. K. Yoshida, Daiichi College of Pharmaceutical Sciences, Fukuoka, Japan. All other chemicals used were of reagent grade.

Results

Comparison of activity levels of alkaline phosphatase and 5'-nucleotidase in the homogenates and plasma membranes

Two marker enzymes for plasma membranes, alkaline phosphatase and 5'-nucleotidase, were determined both in homogenates and plasma membranes prepared from transplantable ascites hepatoma and rat livers with or without treatment of partial hepatectomy or bile duct ligation (Table I). In homogenates, the specific activity of alkaline phosphatase of the hepatoma was about 5- and 50-fold higher than those of the livers from operated rats and of the unoperated, respectively, and similar differences in specific activity were also observed among the plasma membranes prepared from these homogenates. In contrast to the high activity of alkaline phosphatase, 5'-nucleotidase activity found in the hepatoma was much lower than those in the livers.

Effects of pH and L-histidine on the enzyme activity of the hepatoma membrane were examined using 5'-AMP as substrate. The pH activity curve obtained was quite different from the typical pattern of 5'-nucleotidase of the liver membrane, but resembled that of alkaline phosphatase with 5'-AMP as substrate [37]. The effect of L-histidine was inhibitory on the hepatoma enzyme, which was similar with its effect on alkaline phosphatase but different from its stimulatory effect on 5'-nucleotidase of the liver membrane [37]. These results suggest that the enzyme activity of the hepatoma membrane measured with 5'-AMP as substrate was exhibited predominantly by alkaline phosphatase. To know the exact activity level of 5'-nucleotidase in the hepatoma membrane, we carried out the following experiments. In the following results, a term of 5'-AMPase activity is used as a broad definition referring to activity of any enzyme(s) including 5'-nucleotidase which hydrolyzes 5'-AMP irrespective of any pH of the reaction medium used.

TABLE I

COMPARISON OF ACTIVITIES OF ALKALINE PHOSPHATASE AND 5'-NUCLEOTIDASE IN HOMOGENATES AND PLASMA MEMBRANES OF RAT LIVERS AND HEPATOMA AH-130

The specific activities of enzymes were expressed as nmol/min per mg protein (means ±S.D.).

Source	Number of experiments *	Specific activity of alkaline phosphatase		Specific activity of 5'-nucleotidase	
		Homogenate	Plasma membranes	Homogenate	Plasma membranes
Normal liver	12	2 ± 0.8	53 ± 11	44 ± 4	793 ± 81
Regenerating liver **	3	19 ± 3	471 ± 93	48 ± 6	826 ± 93
Bile duct ligated liver **	4	16 ± 4	421 ± 75	45 ± 7	769 ± 103
Hepatoma AH-130 ***	11	106 ± 21	1,445 ± 190	6 ± 2	107 ± 27

* Each experiment was done with 3—5 rats.

** Livers were taken 24 h after each treatment [17,18].

*** Cells were harvested 8—10 days after transplantation.

Effects of lectins on activities of membrane-bound enzymes

5′-Nucleotidase [24] and alkaline phosphatase [25] are glycoproteins, and concanavalin A has been shown to inhibit 5′-nucleotidase activity [26]. Fig. 1a shows effects of concanavalin A, *Ricinus communis* agglutinin, and wheat germ agglutinin on 5′-AMPase activity at pH 7.5 of the liver plasma membrane. The activity was increasingly inhibited by increase of lectin concentrations. Maximum inhibitions exhibited by concanavalin A, *R. communis* and wheat germ agglutinins were 95, 50 and 40%, respectively. These inhibitions were released by the addition of competitive sugars corresponding to each lectin in the reaction mixture, as shown in Fig. 1a. Fig. 1b shows effects of three lectins on 5′-AMPase activity of the hepatoma plasma membrane. As compared with the inhibitions shown in Fig. 1a, the hepatoma enzyme was much less inhibited by concanavalin A, *R. communis* and wheat germ agglutinins, showing maximum inhibitions of about 55, 35, and 10%, respectively. When the influence of these lectins on the enzyme activity after it was extracted with *n*-butanol was examined, the results were almost identical to those found with intact membranes shown in Figs. 1a and 1b.

When effects of the lectins on alkaline phosphatase activity were examined using *p*-nitrophenylphosphate as substrate, no inhibitory effect of the lectins was observed at any pH value, irrespective of the enzyme sources (data not shown).

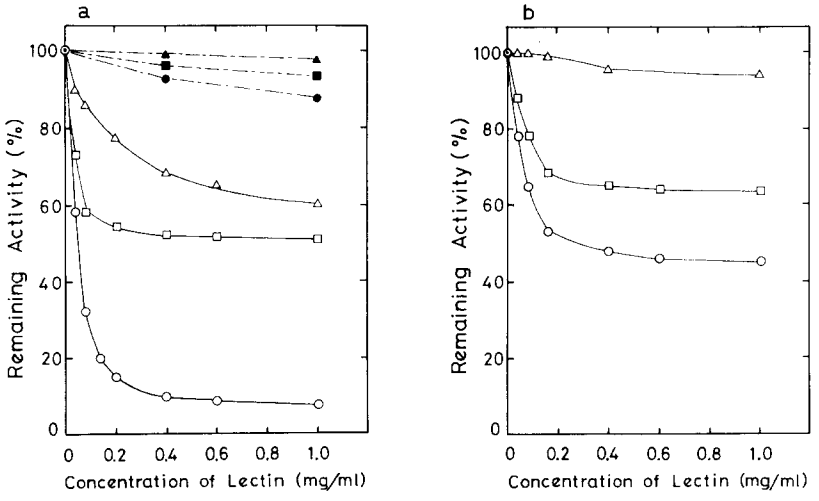
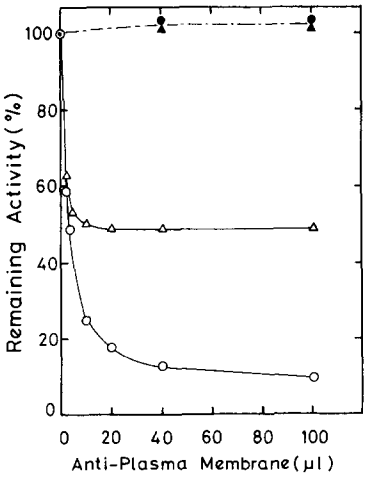


Fig. 1. Effects of lectins on the 5′-AMPase activity of plasma membranes isolated from rat liver (a) and hepatoma AH-130 (b). (a) After plasma membrane (30 μg protein) prepared from rat liver was preincubated for 10 min at 37°C in the reaction medium without substrate in the presence of either concanavalin A (○), *R. communis* agglutinin (□), or wheat germ agglutinin (△) at the concentrations indicated, 5′-AMPase activities were determined at pH 7.5 as described in Materials and Methods. Values are expressed as percentages of the control activity (determined without lectin). Release of an inhibitory action of each lectin was examined by the addition of a competitive inhibitor corresponding to each lectin at the preincubation time; α-methyl-D-glucoside (●) against concanavalin A, D-galactose (■) against *R. communis* agglutinin, and *N*-acetyl-D-glucosamine (▲) against wheat germ agglutinin. The final concentration of these sugars was 0.2 M. (b) Plasma membrane (500 μg protein) prepared from the hepatoma was treated with lectins and then assayed for 5′-AMPase activities at pH 7.5 as described above. Symbols for lectins were the same as in (a).

These results suggest that less inhibition of the hepatoma 5'-AMPase by the lectins shown in Fig. 1b was due to a contribution of alkaline phosphatase measurable even at pH 7.5 because of its high activity in the hepatoma and its capacity for hydrolizing 5'-AMP.

Effects of anti-plasma membrane antisera on activities of membrane-bound enzymes

Fig. 2 shows effects of antiserum produced against the liver plasma membrane on the 5'-AMPase activity. Approximately 90% of 5'-AMPase activity of the liver plasma membrane was inhibited by the addition of 0.1 ml antiserum, while only 50% of activity of the hepatoma enzyme was inhibited by the antiserum. On the other hand, the antiserum did not show any effect on alkaline



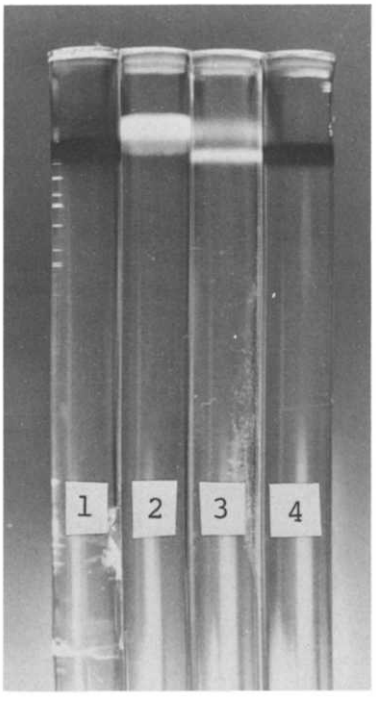


Fig. 2. Effects of anti-liver plasma membrane antiserum on the activities of 5'-AMPase and alkaline phosphatase of plasma membranes isolated from rat liver and hepatoma. Plasma membranes isolated from rat liver (○) and hepatoma (△), 30 μg and 500 μg protein, respectively, were preincubated for 10 min at 37°C in the reaction medium without substrate in the presence of anti-liver plasma membrane antiserum at the indicated volumes, and 5'-AMPase activities were then determined at pH 7.5. The 5'-AMPase activity found in the added antiserum, which had separately been assayed as blank, was subtracted in each determination. Values are expressed as percentage of the control activity which was determined without antiserum. Closed symbols represent the effect of the antiserum on alkaline phosphatase activity which was determined at pH 9.0; rat liver plasma membrane (●) and hepatoma plasma membrane (▲).

Fig. 3. Electrophoretic comparison of activities of 5'-AMPase and alkaline phosphatase extracted from plasma membranes of rat liver and hepatoma. Samples were extracted from both plasma membranes, electrophoresed in polyacrylamide gels, and stained for enzyme localizations as described in Materials and Methods. Samples in gels 1 and 2 are from liver plasma membrane, and those in gels 3 and 4, from hepatoma plasma membrane. Gels 1 and 4 were stained for localization of alkaline phosphatase using 5-Br, 4-Cl-indoxylphosphate as substrate, and gels 2 and 3, for 5'-AMPase using 5'AMP as substrate.

phosphatase activity, as shown in Fig. 2. Furthermore, antiserum produced against the hepatoma plasma membrane inhibited neither activities of 5′-AMPase and alkaline phosphatase from both enzyme sources (data not shown).

From the similarity between the inhibition curves with concanavalin A (Fig. 1) and with anti-liver plasma membrane serum (Fig. 2) it can be concluded that these effectors specifically inhibit 5′-nucleotidase activity and that about 50% of 5′-AMPase activity of the hepatoma membrane measured at pH 7.5 is not activity of 5′-nucleotidase but of alkaline phosphatase which hydrolyzes 5′-AMP.

Electrophoretic comparison of the two enzymes in polyacrylamide gels

Fig. 3 shows localizations of 5′-AMPase and alkaline phosphatase after polyacrylamide gel electrophoresis. When 5′-AMP was used as substrate for activity localization at pH 7.5, two activity bands appeared in both samples from the liver and the hepatoma (gels 2 and 3). When the intensity of two activity bands was compared, in gel 2 of the liver sample the first band with a slower mobility

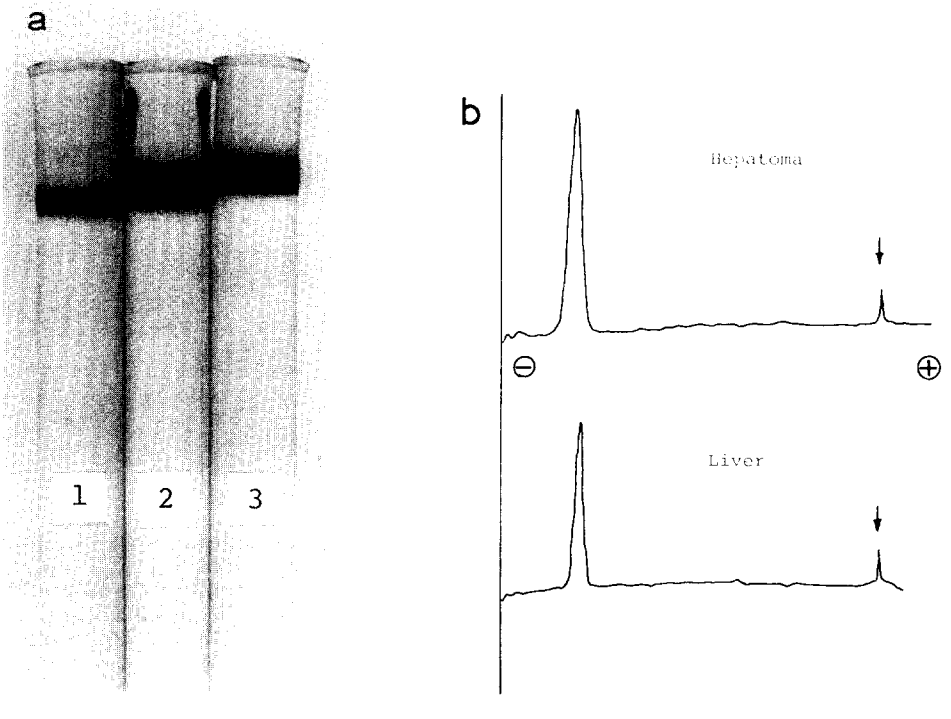


Fig. 4. Electrophoretic comparison of alkaline phosphatases of plasma membranes isolated from rat liver and hepatoma. (a) Samples were extracted from both plasma membranes and electrophoresed in standard polyacrylamide gels followed by staining for localization of alkaline phosphatase activity as described in Materials and Methods. Gel 1, extract of liver plasma membrane; gel 2, mixture of extracts from liver and hepatoma plasma membranes; and gel 3, extract of hepatoma plasma membrane. (b) Plasma membranes of rat liver and hepatoma were solubilized and electrophoresed in the presence of sodium dodecyl sulfate followed by staining for localization of alkaline phosphatase. Gels were scanned at 622 nm. An arrow indicates the position of tracking dye.

was much more intense than the second one, whilst in the gel 3 of the hepatoma sample the second band was more intense than the first. On the other hand, when 5-Br, 4-Cl-indoxylphosphate was used as substrate in the reaction mixture at pH 10.4, only a single activity band was visible in each gel (gels 1 and 4), and the mobility of this band was coincident with that of the second band of 5′-AMPase activity, demonstrating that the first band of 5′-AMPase was stained by 5′-nucleotidase, and the second by alkaline phosphatase.

Comparison of the mobility of each enzyme between the two enzyme sources showed that 5′-nucleotidase of both samples had an identical mobility but alkaline phosphatase from the hepatoma migrated a little slower than that from the liver. The difference of mobility between two alkaline phosphatases was confirmed by the presence of two distinct bands in the mixed sample, as shown in Fig. 4a. The difference, however, was not observed in the sodium dodecyl sulfate gel electrophoresis as shown in Fig. 4b, indicating that the molecular size of the two alkaline phosphatases is identical or very similar. The molecular weight of native alkaline phosphatase was calculated as about 140 000 by co-electrophoresis of standard proteins, IgG, rat serum albumin, and heavy and light chains of IgG.

Discussion

As an alteration of membrane-bound enzymes after malignant transformation of cells, Kim et al. [27] presented evidence suggesting that decrease or absence of 5′-nucleotidase in mammary tumors was correlated with their capacity of metastasis, while Bosmann [28] demonstrated that 5′-nucleotidase activity was significantly elevated in the virus-transformed cells compared with that of the non-transformed control cells. A comparison of the enzyme activities displayed by plasma membranes isolated from rat liver and rat hepatomas was extensively made by Emmelot and Bos [11]. They showed that alkaline phosphatase activity of the hepatoma 484 with *p*-nitrophenylphosphate as substrate increased twice that of the liver, while the 5′-nucleotidase activity of the hepatoma was lower by about 25% than that of the liver. In the present study the activity level of 5′-nucleotidase in the hepatoma membrane was one-fifth as compared with that in the liver membrane (Table I), consisting with the value reported by Réthy et al. [29], who used the same hepatoma AH-130, although they did not show any data on alkaline phosphatase. Activity level of alkaline phosphatase in the ascites hepatoma AH-130 used in the present study was found to be much greater than any value so far reported. Since alkaline phosphatase does not show a strict substrate specificity [30], extremely high activity of alkaline phosphatase might interfere with a specific determination of other enzymes contained in the plasma membrane which hydrolyze a corresponding substrate of phosphate derivatives. In fact, 5′-AMP, which is commonly used as substrate for 5′-nucleotidase, is well hydrolyzed by alkaline phosphatase too, though pH optima are different.

As shown in Figs. 3 and 4, concanavalin A and anti-plasma membrane antiserum proved to be a specific effector discriminating 5′-nucleotidase from alkaline phosphatase, and use of these inhibitors clearly demonstrated that about 50% of 5′-AMPase activity detected in the hepatoma membrane was

exhibited by alkaline phosphatase. Since Riodan and Slavik reported that concanavalin A inhibits 5′-nucleotidase activity [26], the specificity of its effect on 5′-nucleotidase in other tissues has been confirmed by several investigators [31—33]. The inhibition of 5′-nucleotidase by concanavalin A observed in the present study could be ascribed to its direct binding to mannose of carbohydrate chain(s) on the enzyme, since activity of the purified 5′-nucleotidase was also inhibited to the same extent by concanavalin A (unpublished results). In comparison with the effect by lectins of *R. communis* and wheat germ, the substantial effect on the enzyme exerted by concanavalin A suggests importance of mannose residues of the carbohydrate moiety at the active site of the enzyme, although it is not yet clear how the carbohydrate moiety of the enzyme is involved in catalytic activity. On the other hand, the activity of alkaline phosphatase, which is also a glycoprotein and interacts with concanavalin A [25, 34], was not affected by any lectins, indicating that the carbohydrate moiety of this enzyme may be outside the active site.

Similar inhibition of 5′-nucleotidase activity as by concanavalin A was brought about by antiserum produced against the purified plasma membrane from rat liver. As observed with the mouse liver plasma membrane [35], the antiserum did not show any inhibition of other membrane activities tested.

Alkaline phosphatase solubilized from both plasma membranes was shown to have distinctly different mobility upon polyacrylamide gel electrophoresis. Multiple forms of alkaline phosphatase preparations often found in other tissues have been suggested to be based on differences in sialic acid content [36]. Our preliminary experiment also demonstrated that electrophoretic mobility of both enzymes became identical after treatment with neuraminidase. On the other hand, it was demonstrated in our laboratory that sialyltransferase activity in the hepatoma used here had one fifth activity as compared with that found in the normal liver (manuscript in preparation). It is another point of interest to know whether there is any direct relationship between the decreased level of sialyltransferase activity and possible lower content of sialic acid in alkaline phosphatase. Nonetheless, it should be also kept in mind that the possibility of the primary structure of the protein being different for the two enzymes is not yet ruled out. In order to determine any structural difference between the two enzymes, the purification of both enzymes is now in progress.

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