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STUDIES ON 5'-NUCLEOTIDASES OF RAT LIVER

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SUMMARY

Approximately 8% of the 5'-nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) activity (assayed with AMP as substrate at a pH of 7.4 in the presence of 10 mM tartrate) in a homogenate of rat liver behaved as a lysosomal enzyme when the homogenate was fractionated into its subcellular components by differential and density gradient centrifugation. The bulk of the activity sedimented with the nuclear and microsomal fractions. The 5'-nucleotidases in purified lysosomal and plasma membrane fractions exhibited nearly maximal activity at pH values between 7.0 and 9.5, and were similarly influenced by the addition of varying concentrations of Mg^{2+} and Mn^{2+} to the incubation mixture. When a lysosomal fraction solubilized by treatment with 1% Triton X-100 was passed through a Sephadex G-200 column, two peaks possessing 5'-nucleotidase activity appeared in the effluent. The first peak was identical chromatographically to the 5'-nucleotidase associated with plasma membranes and with a lysosomal membrane fraction. The second peak exhibiting 5'-nucleotidase activity contained a lower molecular weight enzyme which could be extracted from lysosomes by 1 mM $NaHCO_3$. Disc electrophoresis of these same fractions on polyacrylamide gel confirmed these chromatographic findings.

INTRODUCTION

The enzyme 5'-nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) is generally assumed to be a rather specific marker for plasma membranes isolated from homogenates of rat liver by a variety of procedures[1–5]. Nevertheless, Widnell and Unkeless[6] in 1968 reported data suggesting that the enzyme is associated with membranes of the endoplasmic reticulum as well as with plasma membranes. In a recent communication[7], Widnell presented further data based on electron microscopic histochemistry substantiating this earlier report; however, a large part of the 5'-nucleotidase in microsomal pellets appeared to be associated with vesicles derived from the plasma membrane. In this laboratory[8], 5'-nucleotidase was used as a marker enzyme

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for plasma membrane fragments in studies involving the preparation of highly purified lysosomes from rat liver. A small part of the 5'-nucleotidase activity of the homogenate was always associated with the most highly purified preparations of lysosomes, and in this report the results of experiments designed to assess the significance of this lysosomal 5'-nucleotidase are presented. A part of this work has been presented in a preliminary form[9].

MATERIALS AND METHODS

Animals

Female Charles River rats, weighing 200–250 g, were used in all experiments. All rats received Purina rat chow and water *ad libitum* until they were fasted overnight before being killed by decapitation. Triton WR-1339 was injected intraperitoneally at a dosage of 85 mg/100 g body weight, 3.5 days before the animals were killed.

Preparation of subcellular fractions

Homogenates of rat liver where fractionated by differential centrifugation into a nuclear fraction, a combined heavy and light mitochondrial fraction, a microsomal fraction, and a supernatant fraction according to the procedure of de Duve *et al.*[10]. Highly purified lysosomes were separated by density gradient centrifugation from the total mitochondrial fraction isolated from the livers of rats pretreated with Triton WR-1339 by the method of Trouet[11] as described by Leighton *et al.*[12]. Fig. 1 gives more details of the procedure. Plasma membranes were prepared either from a microsomal fraction by the method of Touster *et al.*[1] or from a homogenate of rat liver in $NaHCO_3$ according to the procedure of Neville[13].

Enzyme assays

Acid phosphatase (EC 3.1.3.2), glucose-6-phosphatase (EC 3.1.3.9), and 5'-nucleotidase were assayed using a Technicon Autoanalyzer (Technicon Instruments Corporation, Tarrytown, New York). The manifold used for all 3 assays and the reagents for acid phosphatase and glucose-6-phosphatase have been described by Leighton *et al.*[12]. The substrate mixture for the 5'-nucleotidase assay was prepared according to the method of Michell and Hawthorne[14] and contained in a final volume of 1.0 ml: 0.2 ml of 0.05 M AMP (pH 7.4), 0.1 ml of 2 M KCl, 0.1 ml of 0.2 M disodium tartrate, 0.2 ml of 0.1 M $MgCl_2$, 0.25 ml of 0.4 M Tris–HCl buffer (pH 7.4), and 0.15 ml of water. The tartrate was included in the incubation mixture to inhibit lysosomal acid phosphatase[15] and the lysosomal acid nucleotidase described by Arsenis and Touster[16]. Cytochrome oxidase (EC 1.9.1.3) was assayed according to the procedure of Cooperstein and Lazarow[17], and protein determined by the method of Lowry *et al.*[18] with bovine serum albumin as the standard. The activity of 5'-nucleotidase in segments of polyacrylamide gel was measured by the following procedure. Segments of the gel 0.5 cm in length, were dropped into 1.8 ml of a substrate mixture *minus* AMP containing 0.2 ml of 1 M KCl, 0.2 ml of 0.2 M tartrate, 0.25 ml of 0.4 M Tris–HCl buffer (pH 7.4) and 1.15 ml of water and the gels dispersed as finely as possible with a stirring rod. The reaction was started by the addition of 0.2 ml of 0.05 M AMP (pH 7.4) and allowed to proceed at 37 °C for 1 h. 1 ml of 15% trichloroacetic acid was then

added and precipitated proteins as well as gel fragments removed by centrifugation. The inorganic phosphate in the supernatant was measured by the method of Fiske and SubbaRow[19] as adapted to the autoanalyzer by Leighton *et al.*[12].

Chromatographic procedures

Sephadex G-200 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) was swollen in 0.05 M Tris–HCl buffer (pH 8.0) containing 1% Triton X-100 for 24 h at 60 °C. After cooling, the swollen gel was packed into a 2.5 cm × 35 cm column and equilibrated with the same buffer at 4 °C. The column was eluted in the cold at a flow rate of 0.3 ml per min and 2.5-ml fractions collected. The flow rate of the column was controlled by a peristaltic pump (Buchler Instruments, Inc., Fort Lee, N.J.) interposed between the column and the fraction collector.

Disc electrophoresis

The procedure for electrophoresis on polyacrylamide gel was modeled after the procedure of Davis[20] as modified by Dulaney and Touster[21], using a Polyanalyst apparatus (Buchler Instruments, Inc.). The separating gels (5% polyacrylamide) were polymerized in 0.05 M Tris–HCl buffer (pH 8.0) containing 0.1% Triton X-100. The buffer in the upper electrode reservoir also contained 0.1% Triton X-100. The samples of plasma membranes and lysosomes were dissolved in this same buffer containing 1% Triton X-100 and 20% sucrose and then 0.1-ml aliquots carefully layered on top of the stacking gel. Voltage was applied across the gels and current allowed to flow at a rate of 5 mA per gel for 3 h while circulating cold water (2 °C) through the apparatus. At the end of the electrophoresis the gels (0.4 cm × 6 cm) were removed in the cold and cut into 10 segments. The first 9 segments were 0.5 cm in length and the 10th segment approximately 1.5 cm in length. Segment No. 1 extended 0.25 cm on either side of the interface between the stacking and separating gels and thus contained any material which did not migrate into the separating gel.

Chemicals

Disodium β-glycerophosphate (grade I), dipotassium glucose 6-phosphate, cytochrome *c* (type VI), adenosine 5'-monophosphate (type II), Triton X-100 and bovine serum albumin were purchased from Sigma Chemical Co., St. Louis, Mo. Triton WR-1339 was obtained from Ruger Chemical Co., Irvington-on-Hudson, N.Y. All other chemicals were of reagent grade.

RESULTS

After either an intravenous or intraperitoneal injection of Triton WR-1339, this non-hemolytic detergent is taken into the cells of the liver by pinocytosis[22] and eventually accumulates in the lysosomes of both hepatocytes and Kupffer cells. The Triton WR-1339 in the lysosomes decreases their equilibrium density in sucrose gradients and allows their separation from the mitochondria, peroxisomes, and other membranous fragments in a mitochondrial fraction by flotation through a discontinuous sucrose gradient (see Fig. 1). Table I gives a summary of the distributions of 5'-nucleotidase (marker enzyme for plasma membranes), acid phosphatase (marker enzyme for lysosomes), glucose-6-phosphatase (marker enzyme for membranes of the endoplasmic

TABLE I

DISTRIBUTION OF ENZYMES IN SUBCELLULAR FRACTIONS FROM RAT LIVER

For subcellular fractions isolated from liver homogenates by differential centrifugation, percentage values are expressed as percent of recovered liver activity while for the gradient fractions separated from a total mitochondrial fraction values are expressed as percent of recovered gradient activity. Recoveries

$$\left(\frac{N + ML + PS}{E + N}\right) \times 100$$

where E = post-nuclear supernatant, of enzymes and protein in the fractions isolated by differential centrifugation were 94.8 ± 3.2 (mean ± standard deviation), 95.4 ± 5.3, 95.6 ± 7.2, 80.3 ± 8.9 and 93.4 ± 2.5 for 5′-nucleotidase, acid phosphatase, glucose-6-phosphatase, cytochrome oxidase and protein, respectively. Recoveries

$$\left(\frac{\text{Fraction 1} + \text{Fraction 2} + \text{Fraction 3} + \text{Fraction 4}}{ML}\right) \times 100$$

from the gradients were 93.1 ± 2.1, 88.5 ± 4.5, 95.5 ± 19.2, 84.3 ± 11.9 and 88.0 ± 5.6 for 5′-nucleotidase, acid phosphatase, glucose-6-phosphatase, cytochrome oxidase and protein, respectively, using animals pretreated with Triton WR-1339 and 84.3 ± 13.9, 92.7 ± 2.6, 80.9 ± 8.5, 84.7 ± 7.4, and 91.6 ± 3.2, respectively, using animals not pretreated with Triton WR-1339. Numbers in parentheses designate the number of experiments.

Fraction		*Percentage values*				
		5′-Nucleotidase	*Acid phosphatase*	*Glucose-6-phosphatase*	*Cytochrome oxidase*	*Protein*
Nucleair fraction (N)		17.9 ± 1.0 (3)	2.5 ± 0.9 (12)	5.3 ± 2.9 (12)	7.8 ± 2.2 (12)	13.9 ± 1.3 (12)
Heavy *plus* light mitochondrial fraction (ML)		7.8 ± 1.0 (3)	47.5 ± 7.5 (12)	10.4 ± 2.7 (12)	89.8 ± 2.6 (12)	27.5 ± 1.7 (12)
Microsomal *plus* supernatant fraction (PS)		74.3 ± 2.6 (3)	50.1 ± 7.8 (12)	84.3 ± 4.5 (12)	2.4 ± 1.7 (12)	58.6 ± 1.9 (12)
Gradient Fraction 1*	− Triton** WR-1339	0.7 ± 0.9 (3)	0.1 ± 0.0 (3)	0.1 ± 0.1 (3)	0 (3)	0.1 ± 0.0 (3)
	+ Triton WR-1339	0.6 ± 1.1 (3)	2.1 ± 1.6 (12)	0 (12)	0 (12)	0.2 ± 0.1 (12)
Gradient Fraction 2	− Triton WR-1339	1.2 ± 0.6 (3)	1.5 ± 0.5 (3)	0.7 ± 0.6 (3)	0.1 ± 0.0 (3)	0.5 ± 0.1 (3)
	+ Triton WR-1339	31.8 ± 8.6 (3)	62.9 ± 6.7 (12)	5.8 ± 3.7 (12)	0.1 ± 0.0 (12)	3.2 ± 0.6 (12)
Gradient Fraction 3	− Triton WR-1339	2.1 ± 0.3 (3)	0.8 ± 0.2 (3)	0.8 ± 0.4 (3)	0.1 ± 0.1 (3)	0.8 ± 0.2 (3)
	+ Triton WR-1339	3.9 ± 1.2 (3)	6.2 ± 2.5 (12)	0.5 ± 0.8 (12)	0.3 ± 0.2 (12)	1.5 ± 0.8 (12)
Gradient Fraction 4	− Triton WR-1339	95.9 ± 1.7 (3)	97.6 ± 0.6 (3)	98.5 ± 1.1 (3)	99.8 ± 0.2 (3)	98.6 ± 0.4 (3)
	+ Triton WR-1339	63.6 ± 7.2 (3)	28.9 ± 7.3 (12)	93.6 ± 3.8 (12)	99.6 ± 0.2 (12)	95.2 ± 1.2 (12)

* See Fig. 1.

** Heavy *plus* light mitochondrial fraction (ML) subfractionated by density gradient centrifugation was isolated from the livers of rats not pretreated with Triton WR-1339.

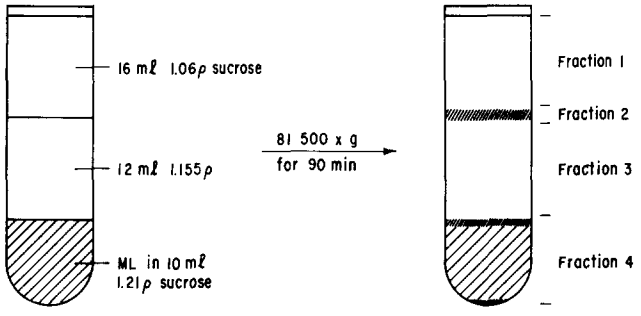

Fig. 1. Purification of lysosomes from rat liver by flotation through a discontinuous sucrose gradient. At the end of the centrifugation period, 1.30 density sucrose was introduced into the bottom of the tube and the fractions collected into chilled tubes through a collecting cone fitted on top of the tube[12]. Highly purified lysosomes collect in Fraction 2 at the interface between the 1.155 and 1.06 density sucrose.

reticulum), cytochrome oxidase (marker enzyme for mitochondria), and protein in the 3 subcellular fractions separated from homogenates of rat liver by differential centrifugation and in the 4 fractions collected from the gradient used for the further purification of lysosomes. The effects of omitting the pretreatment of the rats with Triton WR-1339 on the distributions of the enzymes in the gradient are also summarized. As shown many times previously, pretreatment of the rats with Triton WR-1339 caused a shift of acid phosphatase from Fraction 4 of the gradient into Fraction 2 (the purified lysosomal fraction). When the mitochondrial fraction was isolated from the livers of rats not pretreated with Triton WR-1339, Fraction 2 of the gradient contained 2% of the total gradient acid phosphatase and Fraction 4 98%, as compared to 63% in Fraction 2 and 29% in Fraction 4 when the mitochondrial fraction was isolated from the livers of rats pretreated with Triton WR-1339. Pretreatment with Triton WR-1339 also altered significantly the distribution of 5'-nucleotidase in the gradient. Fraction 2 contained 1% of the total gradient 5'-nucleotidase and Fraction 4 96% when the mitochondrial fraction under study was isolated from the livers of animals not pretreated with Triton WR-1339 as compared to 32% in Fraction 2 and 64% in Fraction 4 when the mitochondrial fraction was isolated from the livers of animals pretreated with Triton WR-1339. The mitochondrial fraction contained only 8% of the total liver 5'-nucleotidase; therefore the purified lysosomal fraction (Fraction 2 of the gradient) contained approximately 2.5% of the total liver 5'-nucleotidase. The bulk of the liver 5'-nucleotidase sedimented with the nuclear and microsomal fractions. When the combined microsomal and supernatant fraction was separated, approximately 80% of the 5'-nucleotidase of the fraction sedimented with the microsomal pellet. The data presented in Table I suggest that a portion of the 5'-nucleotidase in the mitochondrial fraction is associated either with lysosomes or with other vesicles (*e.g.* pinocytotic vesicles or some type of residual body filled with Triton WR-1339) whose densities are also influenced by pretreatment with Triton WR-1339. Therefore it was necessary to study the distribution of 5'-nucleotidase in subcellular fractions free of Triton WR-1339.

The distribution of 5'-nucleotidase in a light mitochondrial fraction and in a slightly modified microsomal fraction isolated from the livers of rats not pretreated

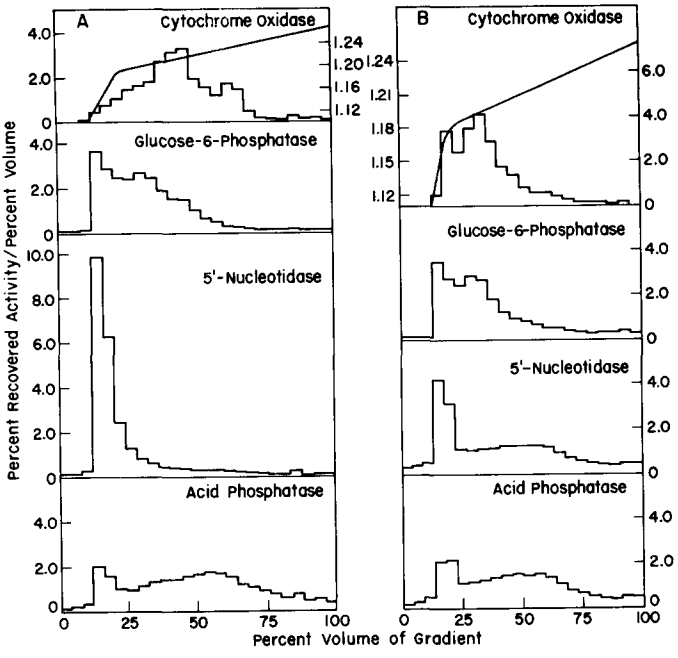

Fig. 2. Fractionation of subcellular particles from rat liver on continuous sucrose gradients. (A) A slightly modified microsomal fraction (sedimented between $2 \cdot 10^4$ and $50 \cdot 10^4 \times g \cdot \min$ in the Spinco 50.1 rotor) from 8.3 g of liver was suspended in 5 ml of 0.25 M sucrose and layered on top of a continuous sucrose gradient extending from a density of 1.15 to 1.25 in a centrifuge tube for the Spinco SW 27 rotor. The tube was centrifuged for 3 h at 25 000 rev./min and then fractions of approximately 1.5 ml collected into tared tubes. The fractions were weighed, their densities measured by refractometry, and their exact volumes calculated. (B) A light mitochondrial fraction from 11.0 g of liver was suspended in 5 ml of 0.25 M sucrose and layered on top of a continuous sucrose gradient extending from a density of 1.15 to 1.27. See legend to (A) for all other details. The densities of the fractions are indicated on the upper graphs.

with Triton WR-1339 was studied by isopycnic centrifugation of the fractions on continuous sucrose gradients. The 5′-nucleotidase in the modified microsomal fraction equilibrated around a density of 1.13, the density expected of plasma membrane fragments in sucrose gradients[1,23] (Fig. 2A); however, slightly more than 50% of the 5′-nucleotidase in a light mitochondrial fraction which was low in microsomal contamination equilibrated at densities greater than 1.19 (Fig. 2B). The acid phosphatase equilibrating in a broad peak around a density of 1.22 in both Figs 2A and 2B is undoubtedly associated with lysosomes since this is the characteristic density profile of lysosomes isolated from the livers of rats not pretreated with Triton WR-1339. The distributions of 5′-nucleotidase and of acid phosphatase in the light mitochondrial fraction gradient were quite similar, and, in fact, the 5′-nucleotidase in fractions with densities greater than 1.19 most likely was associated with lysosomes since only lysosomes from untreated animals equilibrate at such high densities in sucrose gradients[12]. Although the data presented in Table I and Fig. 2 demonstrate that a small portion of the 5′-nucleotidase of the liver is associated with lysosomes, they provide no information concerning the identity or nature of the enzyme(s) in the lysosomal fraction responsible for the activity. Although tartrate was included in the substrate

mixture for the 5′-nucleotidase assay to inhibit acid phosphatase[15] and the acid nucleotidase of lysosomes[16], it is possible that a small residual activity of one of these enzymes or that some other nonspecific lysosomal phosphatase was responsible for the nucleotidase activity observed at a pH of 7.4 in the purified lysosomal preparations. Alternatively, the fusion of pinocytotic vesicles with lysosomes might incorporate 5′-nucleotidase of plasma membrane origin into the membranes of lysosomes. Wattiaux-de Coninck and Wattiaux[24] have presented data indicating that lysosomal membranes may contain the same nucleoside diphosphatase found in the plasma membranes of rat liver and Robinson and Stirling[25] have suggested this possibility for the A form of *N*-acetyl-β-glucosaminidase. Therefore some properties of the 5′-nucleotidase in highly purified preparations of lysosomes and plasma membranes were studied.

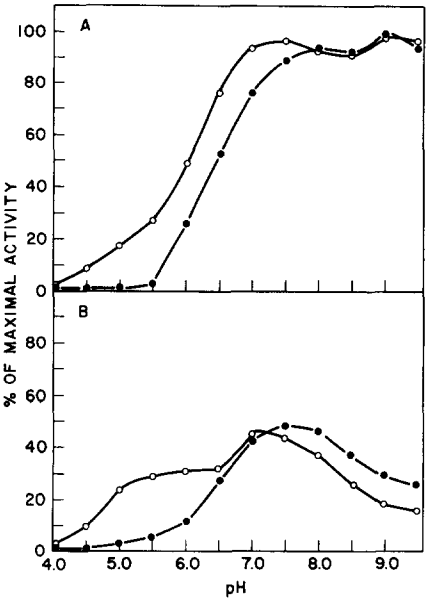

Fig. 3. Activity *versus* pH curves for the 5′-nucleotidase in purified preparations of plasma membranes and lysosomes. (A) These activities were measured in the presence of 10 mM Mg^{2+}. Highly purified lysosomes isolated from the livers of rats pretreated with Triton WR-1339 and plasma membranes isolated by the method of Touster *et al.*[1] were used as sources of enzyme. The lysosomal and plasma membrane fractions were diluted with 1 mM $NaHCO_3$ containing 0.1 g/l of Triton X-100 to a final protein concentration of 85.6 and 40 μg/ml, respectively. A 0.8-ml aliquot of these diluted fractions was used for the assay. The pH values on the abscissa are plotted against % of maximal activity at a pH of 9.0 on the ordinate. (B) These assays were done with no added Mg^{2+} in the presence of 1 mM EDTA. Values on the ordinate are plotted as percent of maximal activity in the presence of 10 mM Mg^{2+}. See (A) for all other details. ○—○, lysosomal fraction; ●—●, plasma membrane fraction.

Fig. 3 shows a pH *versus* activity curve for the 5′-nucleotidase in purified lysosomal and purified plasma membrane fractions. Since Song and Bodansky[26] found two pH optima (pH 7.5 and 9.2) for rat liver 5′-nucleotidase in the presence of Mg^{2+} and only a single optimum (pH 7.5) in the absence of added Mg^{2+}, the pH *versus* activity curves were done in the presence and absence of added Mg^{2+}. The incubation mixtures containing no added Mg^{2+} were also made 1 mM with respect to EDTA in order to

eliminate the possible influence on the enzymatic reaction of endogenous Mg^{2+}. In the presence of Mg^{2+} (Fig. 3A), the enzymes from both sources exhibited maximal activity over a broad pH range from 7.0 to 9.5; however, there was a suggestion of two pH optima for both the lysosomal and plasma membrane 5'-nucleotidase. In the absence of added Mg^{2+} (Fig. 3B), the 5'-nucleotidase activity in the two fractions decreased by approximately 75% at a pH of 9.0 and by approximately 50% at a pH of 7.5. Only a single pH optimum at a pH value near neutrality was observed. In contrast to the plasma membrane fraction, the lysosomal fraction possessed a significant amount of 5'-nucleotidase activity at pH values below 6.0. Examination of the data illustrated in Figs 3A and 3B reveals that the nucleotidase activity of the lysosomal fraction in the acid pH range was not significantly increased by the addition of Mg^{2+}, suggesting that either acid phosphatase or some other lysosomal phosphatase not requiring Mg^{2+} may have been responsible for the hydrolysis of AMP at these acid pH values. A typical plasma membrane fraction released approximately 21 μmoles of inorganic phosphate (P_i) from AMP per mg protein at pH 7.4 using the assay conditions and incubation period employed in this study (assays were done using an autoanalyzer; therefore it is difficult to estimate the exact incubation time) as compared to a release of 5.4 μmoles of P_i per mg protein by the purified lysosomal preparations. The plasma membrane fraction used to prepare these curves was free of contamination by mitochondria (cytochrome oxidase) and lysosomes (acid phosphatase); however, based on glucose-6-phosphatase measurements, the fraction was rather badly contaminated with membranes of the endoplasmic reticulum which undoubtedly lowered the specific activity of 5'-nucleotidase in these preparations. As will be discussed later, this should not influence the results since the 5'-nucleotidase in microsomal and purified plasma membrane fractions are electrophoretically identical; furthermore, the pH *versus* activity curves of the 5'-nucleotidase of crude microsomal pellets are nearly identical to the curves for the 5'-nucleotidase in purified plasma membrane fractions (unpublished results).

The effects of varying concentrations of Mg^{2+} and Mn^{2+} on the activity of the 5'-nucleotidase of lysosomal and plasma membrane fractions are illustrated in Fig. 4. The 5'-nucleotidases in both fractions were maximally activated in the presence of 1 mM Mg^{2+} (Fig. 4B). Song and Bodansky[26] demonstrated that low concentrations of Mn^{2+} (0.1 mM) in their assay system stimulated the 5'-nucleotidase of rat liver and that higher concentrations (1 mM) inhibited the enzyme. Similar results were obtained with the 5'-nucleotidases examined in this study (Fig. 4A); however, the 5'-nucleotidase of the plasma membrane fraction was inhibited to a much greater extent by higher concentrations of Mn^{2+} than was the 5'-nucleotidase in the lysosomal fraction. Although the pH *versus* activity curves for the 5'-nucleotidases in lysosomal and plasma membrane fractions were similar at neutral and alkaline pH values (Fig. 3), and although both activities were also influenced similarly by the presence of Mg^{2+} and Mn^{2+} in the incubation mixture (Fig. 4), the data must be interpreted with caution and cannot be used as evidence for the identity of the enzymes in the lysosomal and plasma membrane fractions since many uncontrolled factors, *e.g.* the environment of the enzymes, could influence these parameters. The environment of the 5'-nucleotidase tightly bound to the plasma membranes is obviously quite different from that of the enzyme associated with lysosomes; therefore, further chromatographic and electrophoretic characterizations of the enzymes were undertaken.

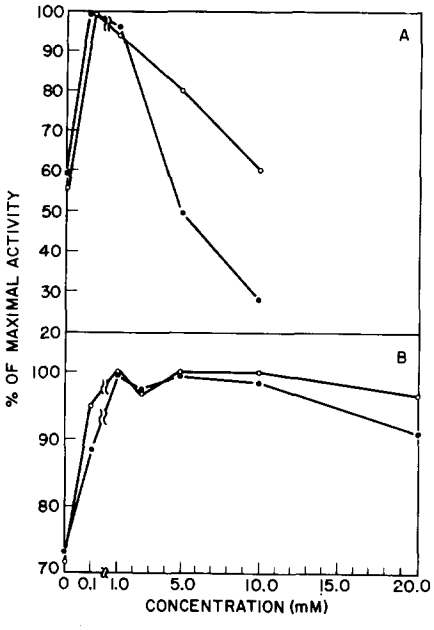

Fig. 4. Effect of Mg^{2+} and Mn^{2+} on the activity of 5′-nucleotidase in lysosomal and plasma membrane fractions. The plasma membrane fraction used in these experiments was prepared by the method of Neville[13]. Dilutions of the lysosomal and plasma membrane fractions containing 44.5 and 17.9 μg of protein/ml were used as enzyme sources. (A) illustrates the effect of Mn^{2+} and (B) the effect of Mg^{2+} on the 5′-nucleotidase in the two fractions. See legend to Fig. 3 for all other details. ○—○, lysosomal fraction; ●—●, plasma membrane fraction.

TABLE II

PARTITION OF ENZYMES AND PROTEIN BETWEEN A SOLUBLE AND A MEMBRANE FRACTION FROM LYSOSOMES AND PLASMA MEMBRANES

The lysosomal pellet isolated from 4.4 g of liver from a rat pretreated with Triton WR-1339 was suspended in 4 ml of ice-cold 1 mM $NaHCO_3$, transferred to a dialysis tubing, and then dialyzed with stirring against 2 l of 1 mM $NaHCO_3$ for 12 h at 4 °C. A pellet containing the plasma membranes from 6.5 g of liver was suspended in 4.0 ml of 1 mM $NaHCO_3$ and dialyzed as above. The particulate material in the dialyzed suspensions was collected by centrifugation at 40 000 rev./min for 30 min in the No. 40 rotor for the Spinco Model L3-50 preparative ultracentrifuge. Values are expressed as percent of recovered activity.

Enzyme or protein	*% Activity*			
	Lysosomal fraction		*Plasma membrane fraction*	
	Pellet	*Soluble fraction*	*Pellet*	*Soluble fraction*
5′-Nucleotidase	29.7	70.3	100	0
Acid phosphatase	58.3	41.7	—*	—
Protein	36.0	64.0	92.8	7.1

* Acid phosphatase was not measured because this fraction exhibited virtually no acid phosphatase activity.

A lysosomal fraction isolated from the livers of rats pretreated with Triton WR-1339 was dialyzed against 1 mM $NaHCO_3$ in the cold, and then separated into a soluble and membrane fraction by the procedure of Trouet[27]. The partition of 5′-nucleotidase, acid phosphatase and protein between these fractions was studied. A plasma membrane fraction was studied in the same manner (Table II). The activity of the enzymes in the fractions was in the range expected on the basis of previous experiments with purified lysosomal and plasma membrane fractions; therefore, the dialysis did not appear to inactivate the enzymes under study. The distributions observed for acid phosphatase and for protein in the lysosomal fraction are very similar to those reported by Trouet[27]. The specific activity of 5′-nucleotidase in the soluble and membrane fractions of the lysosomes can be calculated by combining the data presented in Table II with the data already presented concerning the specific activity of 5′-nucleotidase in purified lysosomal fractions (5.4 μmoles P_i released/mg protein). The estimated specific activities arrived at by this method are 5.9 μmoles P_i released per mg of soluble lysosomal protein and 4.4 μmoles P_i released per mg of lysosomal membrane protein. The amount of soluble acid phosphatase adsorbed onto the lysosomal membranes during the separation procedure is not known; therefore the data do not necessarily mean that 60% of the acid phosphatase of the lysosomes is associated with the lysosomal membrane *in vivo*. The fusion of pinocytotic vesicles with lysosomes does not appear to be the sole mechanism for the incorporation of 5′-nucleotidase into lysosomes since 5′-nucleotidase incorporated into lysosomes by this mechanism would presumably be tightly bound to the lysosomal membrane. The possibility that lysosomal enzymes may digest away a portion of the newly incorporated plasma membrane fragment and thus either release the 5′-nucleotidase into the soluble phase of

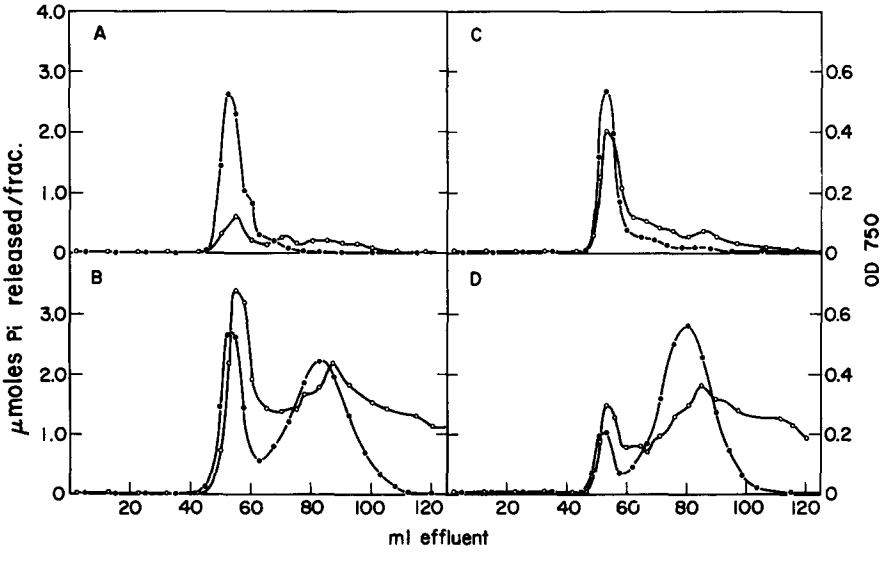

Fig. 5. Chromatography of solubilized subcellular fractions on Sephadex G-200 columns. Samples (1.5 ml) of a solubilized plasma membrane fraction (A), a solubilized lysosomal fraction (B), a solubilized lysosomal membrane fraction (C), and a soluble fraction from lysosomes (D) containing 0.9, 8.6, 2.4 and 6.9 mg of protein, respectively, were applied to the column. See text for all other experimental details. ●—●, 5′-nucleotidase activity; ○—○, protein.

the lysosomes or weaken the bonds between the enzyme and the membrane cannot be eliminated.

A purified lysosomal fraction was prepared by flotation of Triton-filled lysosomes through a discontinuous sucrose gradient and a plasma membrane fraction by the method of Touster *et al.*[1]. In this procedure for the purification of plasma membranes, the livers are homogenized in isotonic sucrose; therefore, these plasma membranes were not subjected to a hypotonic shock in 1 mM $NaHCO_3$ during the isolation procedure. The fractions were dissolved in 0.05 M Tris–HCl buffer (pH 8.0) containing 1% Triton X-100 and aliquots then applied to a Sephadex G-200 column which had been previously equilibrated with the same buffer. In addition, the lysosomal fraction was separated into its soluble and membranous components as described above. The membranes were dissolved in the Tris buffer containing 1% Triton X-100 and then both fractions chromatographed separately on the Sephadex G-200 column. The results are shown in Fig. 5. The 5′-nucleotidase in the plasma membrane fraction chromatographed as a single peak which eluted from the column very near to the void volume (Fig. 5A); however, two peaks with 5′-nucleotidase activity appeared when the solubilized lysosomal fraction was passed through the column (Fig. 5B). The first peak which contained 35% of the activity eluted from the column in the same position as the 5′-nucleotidase in the plasma membrane fraction while the second peak which contained 65% of the activity behaved as a protein of considerably lower molecular weight. The percent distribution between the two peaks was very similar to the distribution observed for 5′-nucleotidase between the soluble and membrane fractions of the lysosomes (Table II) suggesting that the high molecular weight 5′-nucleotidase may represent the membrane-bound form of the enzyme and the lower molecular weight 5′-nucleotidase the soluble form. Furthermore, when the lysosomal membrane

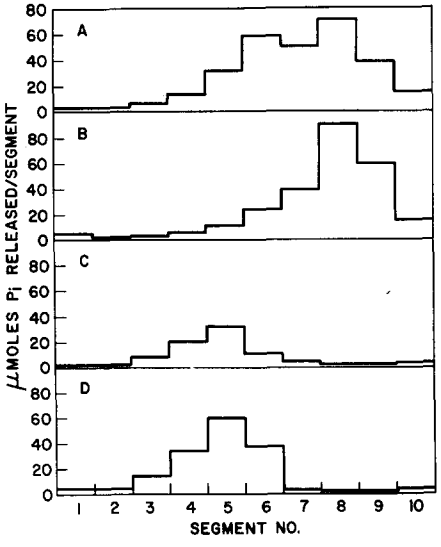

Fig. 6. Electrophoresis of solubilized subcellular fractions on 5% polyacrylamide gel. Samples (0.1 ml) of a solubilized lysosomal fraction (A), a soluble fraction from lysosomes (B), a solubilized lysosomal membrane fraction (C), and a solubilized plasma membrane fraction (D) containing 362, 320, 241 and 234 μg of protein, respectively, were carefully layered on top of the gel. See text for all other experimental details.

fraction was chromatographed on the Sephadex G-200 column, only a single peak of 5'-nucleotidase activity appeared near the void volume of the column (Fig. 5C). The bulk of the 5'-nucleotidase in the soluble fraction of the lysosomes chromatographed as the lower molecular weight enzyme (Fig. 5D). The small peak of activity at the void volume of the column probably reflexes incomplete sedimentation of membranous components in the dialyzed lysosomal extracts by the centrifugation procedure.

These same fractions were subjected to disc electrophoresis on polyacrylamide gels containing 0.1% Triton X-100 and the results are shown in Fig. 6. The fractions were again solubilized in Triton X-100 in preparation for electrophoresis. The 5'-nucleotidase in fractions containing plasma membranes (Fig. 6D) and lysosomal membranes (Fig. 6C) migrated as single bands in the polyacrylamide gels. The enzymes from the two sources had identical mobilities and moved to the midportion of the gel. The lysosomal fraction contained 2 overlapping bands of 5'-nucleotidase activity (Fig. 6A). The faster moving band had a mobility in the gel identical to that of the 5'-nucleotidase in the soluble fraction of the lysosomes (Fig. 6B). Although the slower moving band of 5'-nucleotidase in the lysosomal fraction migrated slightly farther into the gel than the 5'-nucleotidase of the plasma and lysosomal membranes, it seems likely that it is the same enzyme.

DISCUSSION

The data shown in Table I and Fig. 1 clearly demonstrate that a certain part of the 5'-nucleotidase activity in homogenates of rat liver has the sedimentation properties and characteristic density in sucrose gradients of a lysosomal enzyme under varying experimental conditions. A comparison of the recoveries of acid phosphatase and of 5'-nucleotidase in highly purified lysosomal fractions (Table I) shows that approximately 8% of the 5'-nucleotidase in liver homogenates behaves as a lysosomal enzyme. A similar percentage can be arrived at from the data in Fig. 2. The assumption is made that the two enzymes are homogeneously distributed among all lysosomes. These data simply indicate that 8% of the liver 5'-nucleotidase as measured in these experiments (activity based on the release of inorganic phosphate from AMP at a pH of 7.4 in the presence of 10 mM tartrate) has the properties of a lysosomal enzyme in centrifugal fields and sucrose density gradients, and yield no information concerning the identity of the enzyme(s) responsible for the activity. If other assay procedures were used, the results might be quite different. The data of Widnell and associates[6,7] demonstrating that the membranes of the endoplasmic reticulum contain 5'-nucleotidase and those presented in this paper demonstrate that the 5'-nucleotidase activity in liver homogenates is not associated exclusively with fragments of the plasma membrane; however, on a quantitative basis the plasma membrane fragments probably do contain the major part of the activity. These facts should be considered when using 5'-nucleotidase as a marker enzyme for the plasma membranes of rat liver.

The results presented in Figs 5 and 6 concerning the chromatographic and electrophoretic properties of the 5'-nucleotidases in purified lysosomal and plasma membrane fractions illustrate, even more clearly, the complexity of the system. The lysosomal fraction contained two 5'-nucleotidases which were chromatographically and electrophoretically distinct. One of these enzymes was associated with the lysosomal membrane fraction isolated by the procedure of Trouet[27], and the other with

the soluble fraction of the lysosomes. The chromatographic and electrophoretic properties of the 5′-nucleotidase associated with the lysosomal membrane fraction were identical to those of the enzyme associated with purified plasma membranes. Although the data suggest that lysosomes contain two 5′-nucleotidases, *i.e.* different enzymes capable of catalyzing the hydrolysis of AMP, the possibility does exist that the higher molecular weight form of 5′-nucleotidase may be an artifact resulting from the incomplete solubilization of the membrane bound enzyme. The enzyme associated with lysosomal and plasma membranes may not be dispersed in a molecular form by treatment with Triton X-100. The 5′-nucleotidase in solubilized lysosomal membranes and plasma membranes did not accumulate at the interface between the stacking and separating gels during electrophoresis, and in fact migrated rather rapidly in the 5% polyacrylamide separating gel suggesting that the enzyme was not associated with fragments of undissolved membrane. Widnell and Unkeless[6] purified the 5′-nucleotidase of rat liver to the point where the specific activity of the purified enzyme was 400 times greater than the specific activity of the enzyme in crude microsomal pellets. This purified enzyme was associated with only one phospholipid, sphingomyelin; however, the enzyme preparation contained approximately equal amounts of protein and phospholipid. When chromatographed on a column of 6% agarose, this purified 5′-nucleotidase also eluted near the void volume of the column; however, it is difficult to assess the influence of the phospholipid in the preparation on the behavior of the enzyme on this hydrophilic gel.

If the lysosomes do contain two 5′-nucleotidases, the effects of Mg^{2+} and Mn^{2+} concentrations on the activities of the enzymes and the pH *versus* activity curves should be done on the individual enzymes rather than on the whole lysosomal fraction. These experiments have not been undertaken. It is very unlikely on the basis of the pH *versus* activity data (Fig. 3) that a lysosomal phosphatase with an acid pH optimum is responsible for the nucleotidase activity observed at a pH of 7.4 in the lysosomal preparations; furthermore, no inorganic phosphate was released from β-glycerophosphate by these lysosomal fractions at a pH of 7.4 in the presence of 10 mM tartrate demonstrating that acid phosphatase is totally inactive under these conditions. The question as to whether lysosomes, particularly the lysosomal membrane, contain the same 5′-nucleotidase found in plasma membranes remains unanswered. The data indicate that the 5′-nucleotidase in the soluble fraction of the lysosomes is probably distinct from the 5′-nucleotidase associated with the plasma membrane; however, more specific methods, perhaps immunological, will have to be applied to the problem before a final answer can be given. The 5′-nucleotidase in the lysosomal membrane fraction is chromatographically and electrophoretically identical to the 5′-nucleotidase in purified plasma membranes; however, in this case it is impossible to rule out the possibility that the lysosomal membrane fraction may be slightly contaminated with fragments of the plasma membrane.

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