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STUDIES ON THE SOLUBLE AND LYSOSOMAL NEURAMINIDASES OF RAT MAMMARY GLANDS

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SUMMARY

1. Rat mammary glands contain a soluble neuraminidase (*N*-acetylneuraminate glycohydrolase, EC 3.2.1.18) in the cytosol and a particulate neuraminidase strongly bound to the lysosomes. The pH optima were 5.8 for the soluble and 4.4 for the lysosomal enzyme.

2. Cu^{2+}, Hg^{2+} and Zn^{2+} inhibited the soluble but they did not affect the lysosomal neuraminidase. Li^{+}, Na^{+} and K^{+}, on the other hand, inhibited the lysosomal but not the soluble enzyme. Addition of Triton X-100 caused mild stimulation of the soluble neuraminidase and strong inhibition of the lysosomal enzyme.

3. Ovine submaxillary glycoprotein is hydrolyzed by the lysosomal but not by the soluble neuraminidase. However, sialoglycopeptides, isolated from a pronase digest of ovine submaxillary glycoprotein, proved to be a good substrate for both enzymes. Neuramin–lactose sulfate was hydrolyzed at a higher rate than neuramin–lactose by both neuraminidases. Mixed brain gangliosides, the poorest of the substrates tested, were hydrolyzed by the soluble enzyme at a rate proportionally lower than that obtained with the lysosomal neuraminidase.

4. Although a light and a heavy lysosome-rich fraction have been separated from rat mammary gland homogenates, no differences have been detected between the properties of the neuraminidase activity associated with these two fractions.

INTRODUCTION

The supernatant fraction of homogenates of rat mammary glands in isotonic KCl is known to contain a soluble neuraminidase[1–3] with pH optimum 5.8.

A particle-bound neuraminidase, pH optimum 4.4, has now been detected in lysosome-rich fractions obtained from these glands.

Prompted by our recent findings in rat liver preparations[4] a comparative study of the properties of the soluble and particulate mammary neuraminidases was conducted. The results obtained indicate the presence of two different neuraminidases

in rat mammary glands, one that occurs in soluble form in the cytosol, and another firmly bound to the lysosomal particles.

Unlike other lysosomal hydrolases, neuraminidase is very labile and it is easily inactivated by freezing and thawing and by detergents commonly used to solubilize these enzymes.

This work was reported in part at the 54th Annual Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, N.J., April, 1970 (ref. 5).

EXPERIMENTAL

Materials

Neuramin–lactose and neuramin–lactose sulfate were isolated from rat mammary gland extracts and purified as described earlier[6]. Ovine submaxillary glycoprotein was kindly provided by Dr. Alfred Gottschalk, Max Planck Institut, Tübingen, West Germany. Sialoglycopeptides were isolated from pronase digests of ovine submaxillary glycoprotein[7]. β-Glycerophosphate, *p*-nitrocatechol, *p*-nitrocatechol sulfate, *N*-acetylneuraminic acid, phenolphthalein–glucuronide and acridine orange were products of Sigma, St. Louis, Mo. Fluorochemical FC-77 was purchased from Minnesota Mining and Manufacturing Co., St. Paul, Minn. All other chemicals utilized were of the highest purity commercially available.

Sprague–Dawley albino rats (250–300 g) fed on commerical pellet diet, were used for this work. Nursing rats in late lactation, 14–20 days after parturition, or 1–4 days after weaning, were stunned and killed by decapitation. The mammary glands, both inguinal and pectoral, were quickly removed and chilled in ice-cold homogenizing solution. Connective tissue, fat and blood clots were removed, and the glandular tissue was blotted, weighed, minced finely with sharp scissors and homogenized as described below. All centrifugations were done in a Spinco Model L with rotor No. 30 except for centrifugations at 105000 $\times$ *g* which were done with rotor No. 40. For sucrose gradient centrifugations a Beckman L2-65 ultracentrifuge with SW-25.1 rotor were used. All operations were conducted at 0–4° unless stated otherwise.

Methods

Enzyme assays. Neuraminidase was determined by the method described earlier[4]. The incubation mixture contained 400 nmoles of neuramin–lactose as substrate and 30 μmoles of acetate buffer in a total volume of 0.4 ml. The pH of the reaction mixture and the length of the incubation varied according to the enzyme preparation being assayed. The lysosomal enzyme was incubated at 37° for 1 h at pH 4.4 while the soluble enzyme was incubated for 3 h at pH 5.8. In the experiments where the incubations were carried out in presence of 0.25 M sucrose, the incubation mixtures were subjected to ion-exchange chromatography[8] in order to eliminate interfering sucrose prior to the thiobarbituric acid assay[9]. Arylsulfatase (EC 3.1.6.1) was assayed according to the method of ROY[10] using *p*-nitrocatechol sulfate as the substrate. β-Glucuronidase (EC 3.2.1.31) and acid phosphatase (EC 3.1.3.2) were determined according to the methods described by GIANETTO AND DE DUVE[11] and BERTHET AND DE DUVE[12], respectively. One unit of neuraminidase activity is defined as

the amount of enzyme that causes the hydrolysis of 1 nmole of neuramin–lactose per h under the conditions of the assay. The unit of activity for the other hydrolases is 1000 times larger, *i.e.* 1 μmole of substrate per h. All specific activities are expressed as units of enzyme activity per mg of protein. The protein content of the enzyme preparations was determined by the method of LOWRY *et al.*[13] with crystalline bovine serum albumin as the standard.

Preparation of lysosome-rich subcellular fractions. The mammary glands from 3 rats were homogenized in 3 vol. of ice-cold 0.25 M sucrose (all sucrose solutions were prepared in glass-distilled water and they contained 1 mM EDTA (disodium salt)). The homogenization was done in a Waring blendor running at top speed for 1 min. The homogenate was centrifuged at about 2900 rev./min for 10 min. The supernatant fraction was carefully decanted and filtered through glass wool in order to remove congealed lipid and the sediment was homogenized in 1.5 vol. of sucrose solution and centrifuged as above. The process was repeated for a third time and the pooled supernatant fractions were diluted with 0.25 M sucrose to obtain a 1:6 dilution (w/v). The cytoplasmic extract was then centrifuged at 25000 rev./min for 8.5 min and the supernatant fraction was poured off. The sediment was further fractionated by flotation through a discontinuous gradient of sucrose with the use of a scaled down version of the technique of TROUET[14]. The pellets were suspended in 45% (w/w) sucrose (density 1.21) to a total volume of 30 ml. 10-ml portions of this suspension were introduced in the bottom of heavy walled plastic tubes and then 10 ml of 34.5% (w/w) sucrose (density 1.155) and 5 ml of 14.3% (w/w) sucrose (density 1.06) were carefully layered on top of the particulate suspension. The tubes were then placed in the SW-25.1 rotor and centrifuged for 2 h at 25000 rev./min. Two layers of lysosome-rich particles were observed at the interfaces 1.06–1.155 and 1.155–1.21 density, respectively (see Fig. 1, Fractions I and II). These two fractions, were collected separately with the use of a Beckman Fraction Recovery System by introducing FC-77 oil into the bottom of the tubes. The individual fractions, volume about 2 ml, were diluted to 30 ml with 0.25 M sucrose or with distilled water (identical results) and the suspension was centrifuged for 30 min at 25000 rev./min to obtain the pelleted lysosome-rich fractions.

When all four fractions (see Fig. 2) were needed, the layers were carefully decanted with a syringe, I and II were diluted with 0.25 M sucrose, III was diluted with an equal volume of distilled water and IV was suspended in 0.25 M sucrose. After centrifuging at 25000 rev./min for 30 min, suspensions of these pellets (5 g of initial wet tissue per ml) were prepared in distilled water (for neuraminidase) or in 0.25 M sucrose (for the other hydrolases) and the enzymes were assayed as described above.

Ultrastructure studies. The pellets obtained from the lysosome-rich Fractions I and II and from Fraction III were fixed in 2% glutaraldehyde and post-fixed with OsO_4. After washing with buffered neutral formalin, the material was dehydrated with ethanol and embedded in araldite. Sections of these pellets were examined in a Hitachi HU-11B electron microscope.

Histochemical demonstration of lysosomal arylsulfatase by electron microscopy was done by the method of GOLDFISCHER[15].

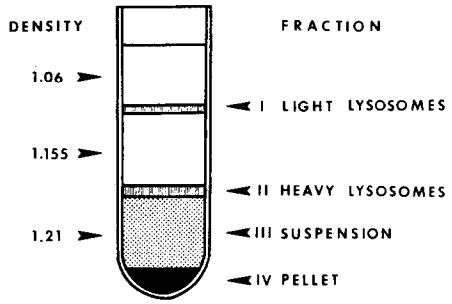


Fig. 1. Distribution of the subcellular particles of rat mammary glands. The fractionation was achieved by flotation through a discontinuous gradient of sucrose as described in the text.

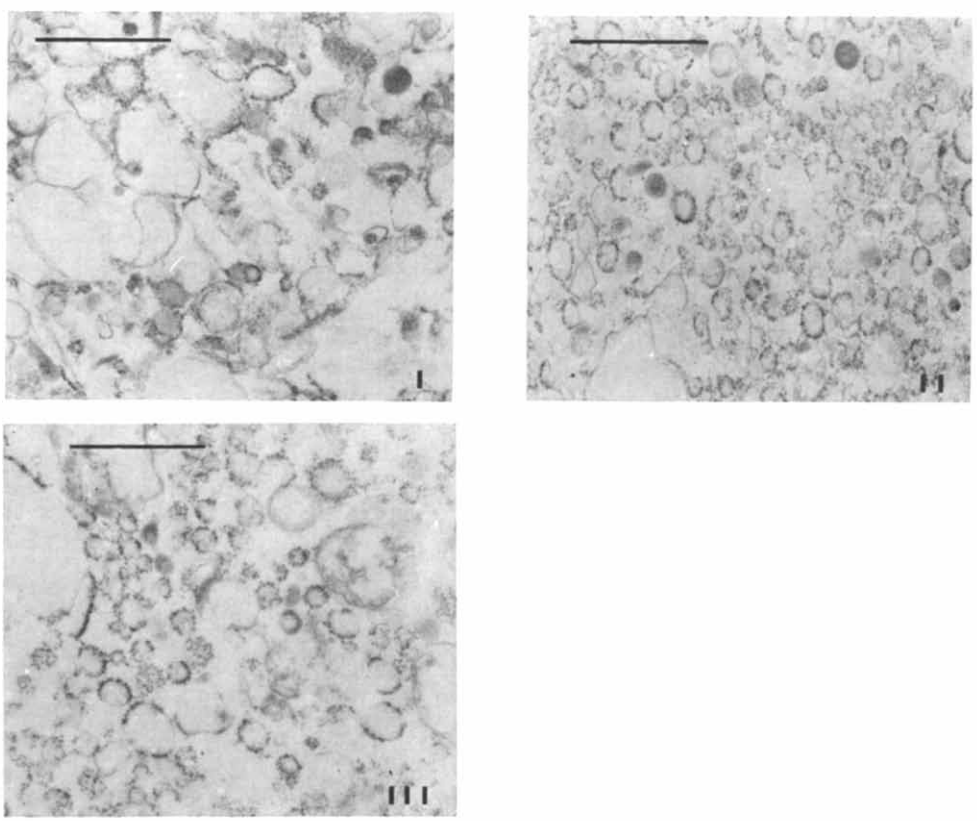


Fig. 2. Electron micrographs of sections through pelleted Fractions I, II and III of rat mammary glands (see Fig. 1). The bar represents 1 μ.

RESULTS

Distribution of particle-bound acid hydrolases

Most of the neuraminidase activity was found in Fractions I and II, less than 20% of the enzyme was usually recovered in Fraction III and no activity was detected in the pellet (Table I). Similar results were obtained with arylsulfatase and β-glucuronidase. The specific activities of these three enzymes were always highest in Fraction II. On occasions, some variability has been observed in the percentage of the total activity of these enzymes recovered in Fraction III. However, the specific activity of these enzymes was, without exception, lower in Fraction III than in either Fraction I or Fraction II. The assay for acid phosphatase in these fractions showed only traces of activity and a different distribution, with most of the enzyme present in Fractions III and IV (Table I).

The electron micrographs of Fractions I, II and III showed the presence of electron-dense bodies. In addition, Fraction I contained smooth vesicles, cysternae

TABLE I

DISTRIBUTION OF ACID LYSOSOMAL HYDROLASES IN PARTICULATE FRACTIONS OF RAT MAMMARY GLANDS

Subcellular particles isolated from a rat mammary gland homogenate, were fractionated by flotation through a discontinuous gradient of sucrose (Fig. 1) and the enzyme assays were conducted on suspensions of the fractions.

Fraction	*Neuraminidase*		*Arylsulfatase*		*β-Glucuronidase*		*Acid phosphatase*	
	Specific activity	*Total units*	*Specific activity*	*Total units*	*Specific activity*	*Total units*	*Specific activity*	*Total units*
I. Light lysosomes	15.3	210	1.6	22	1.0	14	0.3	4
II. Heavy lysosomes	20.0	559	2.8	78	2.4	67	0.4	11
III. Suspension	1.3	155	0.2	24	0.2	24	0.3	36
IV. Pellet	0	0	0	0	0	0	0.1	24

and small amounts of rough membranes, while Fraction II contained mainly small rough-membrane vesicles. Fraction III resembled Fraction II although it contained fewer electron-dense bodies and occasional mitochondria (Fig. 2). The histochemical assay for arylsulfatase in Fractions I and II gave positive results localized in the electron-dense bodies of both pellets. The presence of lysosomes in Fractions I and II was further established by fluorescence microscopy. Typical spherical particles with strong orange fluorescence were observed when the pellets, suspended in 0.25 M sucrose, were treated with acridine orange[16] and examined in a Leitz fluorescence microscope under ultraviolet light (360 nm).

Effect of lysosome-solubilizing agents on bound neuraminidase

When light lysosomes (Fraction I) and heavy lysosomes (Fraction II) were suspended in distilled water, 5–10% of the total neuraminidase activity remained in the supernatant fraction after centrifuging for 1 h at 105000 × *g* (Table II). Freezing and thawing of the aqueous suspensions lead to a progressive loss of activity with only moderate increase of solubilized neuraminidase (10–15% of the total initial value).

TABLE II

EFFECT OF LYSOSOME-SOLUBILIZING AGENTS ON RAT MAMMARY GLAND NEURAMINIDASE

Lysosomes suspended in distilled water were subjected to various treatments and then centrifuged for 1 h at 105 000 × *g*. Neuraminidase activity and protein content were then measured in the supernatant fraction and in the resuspended pellet.

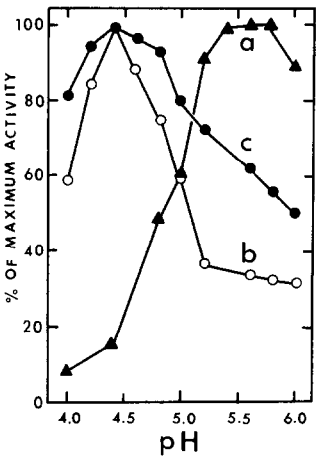
Treatment	*Light lysosomes*				*Heavy lysosomes*			
	Neuraminidase		*Protein*		*Neuraminidase*		*Protein*	
	Activity (units)	*Recovery (%)*	*Content (mg)*	*Recovery (%)*	*Activity (units)*	*Recovery (%)*	*Content (mg)*	*Recovery (%)*
a. Control								
Initial suspension	22.84	100.00	1.31	100.00	42.12	100.00	1.57	100.00
Supernatant	2.10	9.19	0.09	7.03	2.02	4.80	0.04	2.57
b. Freezing and thawing twice								
Pellet	18.49	80.95	1.19	91.07	38.47	91.33	1.48	94.36
Supernatant	3.48	15.24	0.14	10.49	4.86	11.54	0.13	8.19
c. Freezing and thawing 10 times								
Pellet	12.79	60.00	1.16	88.67	21.06	50.00	1.37	87.37
Supernatant	3.48	15.24	0.14	10.95	4.21	9.99	0.20	12.52
d. Sodium deoxycholate (0.1% final concn.)								
Pellet	15.65	68.52	0.41	31.57	22.76	54.06	0.56	35.44
Supernatant	3.88	16.99	0.88	67.39	4.45	10.57	0.88	56.11
e. Triton X-100 (0.05% final concn.), freezing and thawing twice								
Pellet	16.84	73.73	0.76	58.29	30.78	73.08	1.10	69.90
Supernatant	0.64	2.80	0.47	35.83	2.83	2.83	0.38	24.33

Sodium deoxycholate, even at low concentrations (0.1% (w/v)), caused marked inhibition of the enzyme; the non-sedimentable fraction accounted for only 10–17% of the total initial activity (Table II). Freezing and thawing in the presence of 0.05% Triton X-100 gave the lowest yield of solubilized enzyme. Since the levels of activity of the solubilized lysosomal neuraminidase were very low, all the studies were performed with suspensions of the lysosome-bound enzyme. It must be pointed out, however, that the solubilized lysosomal enzyme had consistently higher activity at pH 4.4 than at pH 5.8.

Comparative study of the soluble and lysosomal neuraminidases

Soluble neuraminidase was measured in the supernatant fraction of rat mammary gland homogenates (1:4, w/v) in distilled water or in 0.25 M sucrose, subjected to ultracentrifugation for 1 h at 105000 × *g*. The lysosome-bound neuraminidase was studied in suspensions of light and heavy lysosomes in distilled water or in 0.25 M sucrose.

Kinetic Studies. Soluble neuraminidase showed optimum activity between pH 5.4 and 5.8 with either one of the preparations mentioned above. The pH–activity curve for the soluble fraction obtained from a homogenate in distilled water is shown in Fig. 3; very low activity was detected between pH 4.0 and 4.4. The bound neuraminidase in both light and heavy lysosomes had a pH optimum of 4.4; at pH 5.8 only 30–50% of the maximum activity was detected (Fig. 3). The rate of hydrolysis of



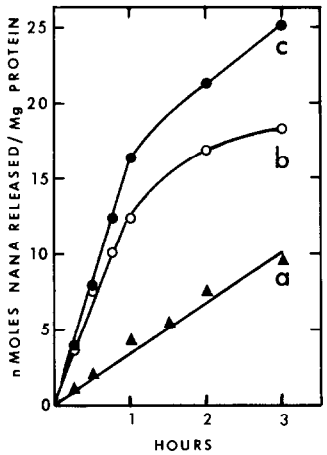


Fig. 3. pH curves of rat mammary gland neuraminidases. Curve a, supernatant fraction (105 000 × *g*, 1 h) of rat mammary gland homogenate (1:4, w/v) in distilled water (4.053 mg of protein per sample); Curve b, suspension of light lysosomal fraction in distilled water (0.613 mg of protein per sample); Curve c, suspension of heavy lysosomal fraction in distilled water (0.916 mg of protein per sample). The pH values shown were obtained with sodium acetate–acetic acid buffers (75 mM final concentration).

Fig. 4. Progress curves of rat mammary gland neuraminidases. Curve a, soluble neuraminidase (same as in Fig. 3), assayed at pH 5.8; Curves b and c are suspensions of the light and heavy lysosomal fractions, respectively, assayed at pH 4.4. NANA, *N*-acetylneuraminic acid.

neuramin–lactose is linear for 3 h with the soluble enzyme while with the lysosomal preparations a decrease in rate was observed after 60 min of incubation (Fig. 4). A linear relationship was observed between protein concentration and *N*-acetylneuraminic acid released with both the soluble and lysosomal preparations. The K_m values, calculated from the $[S]/v$ *versus* $[S]$ plot[17], are $1.75 \cdot 10^{-3}$ M for the soluble neurami-

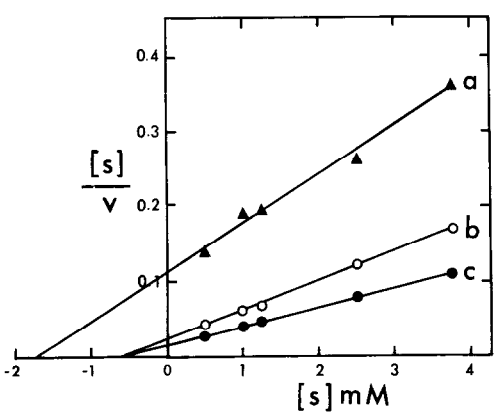
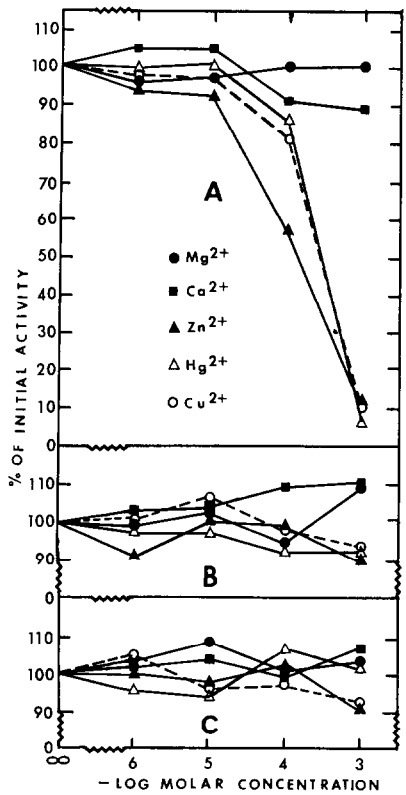


Fig. 5. Effect of substrate concentration on the rate of hydrolysis. Curves a, b, and c correspond to the soluble fraction and the light and heavy lysosomal fractions, respectively. Assays conducted as described under *Methods* except that the concentration of neuramin–lactose was varied as shown.

nidase, and $5.54 \cdot 10^{-4}$ and $5.90 \cdot 10^{-4}$ M for the light and heavy lysosomal fractions respectively (Fig. 5).

Stability. Storage of soluble neuraminidase at 0–4° for 24 h or frozen at −20° for 1 week resulted in only a slight loss of activity. Pelleted lysosomal fractions could be kept frozen at −20° for over a month without appreciable loss of activity. Lysosomal suspensions in 0.25 M sucrose underwent only slight loss of activity after 8 h at 0–4°, while dialysis against distilled water for the same length of time and at the same temperature resulted in 50% loss of neuraminidase activity. Aqueous suspensions of lysosomal fractions lost 10–20% of activity after a week at −20°; successive freezing and thawing resulted in progressive loss of enzyme activity (Table II).

Effect of preincubation. Considerable loss of neuraminidase activity was observed when either the soluble or the lysosomal preparations were incubated at 37° prior to assay. After 10 min, the soluble neuraminidase lost only about 6% of the initial activity whereas losses between 20 and 30% occurred in the lysosomal sus-



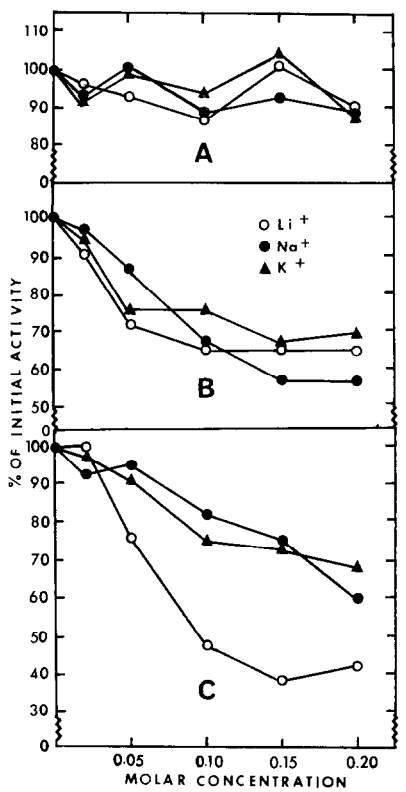


Fig. 6. Effect of bivalent cations on rat mammary gland neuraminidases. The cations (as chlorides) were added to the enzymes and the mixtures were kept for 15 min at 0° prior to the neuraminidase assays. A. Soluble neuraminidase from homogenate in distilled water. B. Light lysosomal fraction suspended in distilled water. C. Heavy lysosomal fraction suspended in distilled water.

Fig. 7. Effect of monovalent cations on rat mammary gland neuraminidases. The cations (as chlorides) were added to the enzyme preparations which were kept for 30 min at 0° prior to enzyme assay. A. Soluble neuraminidase in distilled water. B. Light lysosomal fraction suspended in distilled water. C. Heavy lysosomal fraction suspended in distilled water.

pensions. Soluble neuraminidase retains about 80% of the initial activity after 30 min of preincubation whereas only 50 and 30% of the initial activity were recovered from the suspensions of heavy and light lysosomes, respectively.

Effect of cations. The effect of several bivalent cations (Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+} and Hg^{2+}) at concentrations $1 \cdot 10^{-3}$–$1 \cdot 10^{-6}$ M was studied with the use of their respective chlorides. The results are summarized in Fig. 6. Ca^{2+} and Mg^{2+} had very little effect on soluble or bound neuraminidase. Zn^{2+}, Hg^{2+} and Cu^{2+} caused a marked inhibition of the soluble neuraminidase. At a concentration of $1 \cdot 10^{-3}$ M these three ions inhibit about 90% of the soluble enzyme. These three ions, under identical conditions, did not have any significant effect on either one of the lysosome-bound neuraminidases.

Addition of the monovalent cations Li^+, Na^+ and K^+, as their chlorides, had no effect on the soluble enzyme at concentrations between $2 \cdot 10^{-1}$ and $2 \cdot 10^{-2}$ M. On the other hand, the neuraminidase bound to both lysosomal fractions, was inhibited by these cations. At a concentration of $2 \cdot 10^{-1}$ M between 30 and 60% inhibition was observed (Fig. 7). Mammary gland lysosomes differ from rat liver lysosomes[18] in that no stimulation of bound neuraminidase activity was detected upon prior incubation of either light or heavy lysosomes with $2 \cdot 10^{-2}$ M KCl for 2 h at 0°.

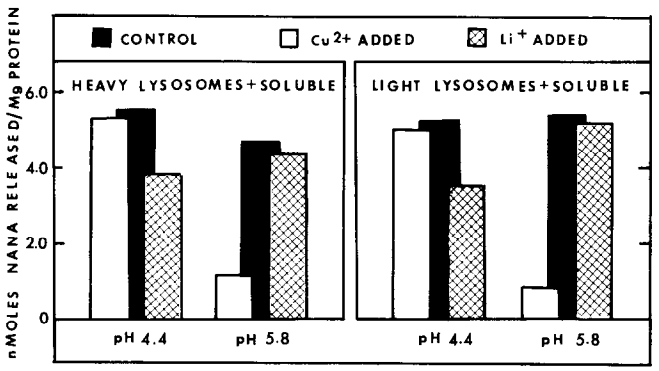


Fig. 8. Effect of Cu^{2+} and Li^+ on mixtures of soluble and lysosomal neuraminidases of rat mammary glands. The light and heavy lysosomal fractions were suspended in soluble enzyme (supernatant fraction of tissue homogenate in distilled water) and the neuraminidase activity of the mixture was measured at pH 5.8 and 4.4 (pH optima for the soluble and lysosomal enzyme, respectively) .The assay was conducted in the presence of 1 mM $CuCl_2$, or 0.15 M KCl, or without additions (control). Incubation time 1 h. NANA, *N*-acetylneuraminic acid.

The specific inhibitory effect of Cu^{2+} and Li^+ on the soluble and the lysosome-bound neuraminidases, respectively, was clearly shown with mixtures of these enzymes, containing either light or heavy lysosomes suspended in soluble neuraminidase. Addition of 1 mM $CuCl_2$ resulted in a decrease of the neuraminidase activity measured at pH 5.8 (soluble neuraminidase), whereas little or no inhibition was observed in the neuraminidase activity of the mixture assayed at pH 4.4 (lysosomal neuraminidase). Conversely, addition of 0.15 M LiCl caused a decrease of the neuraminidase activity measured at pH 4.4 but resulted in insignificant changes of the activity at pH 5.8 (Fig. 8).

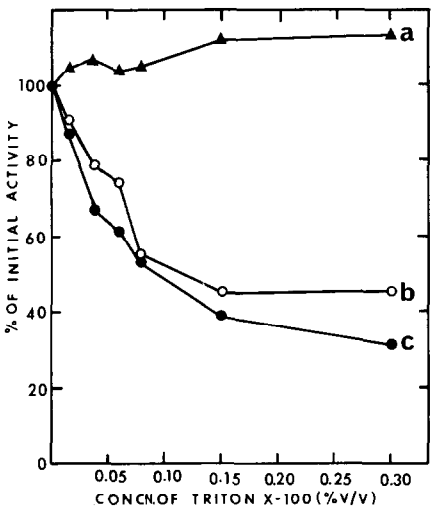


Fig. 9. Effect of Triton X-100 on the neuraminidases of rat mammary glands. Curve a, aqueous soluble neuraminidase; Curve b, light lysosomal fraction suspended in distilled water; Curve c, heavy lysosomal fraction suspended in distilled water. Triton X-100 was added to the enzymes and the mixtures were kept for 30 min at 0° prior to neuraminidase assay.

Effect of Triton X-100. The soluble neuraminidase of rat mammary gland appears to be slightly activated by Triton X-100 (Fig. 9). On the other hand, increasing inhibition of the lysosome-bound neuraminidases was observed with increasing concentrations of this detergent. At a final concentration of 0.3% (v/v), between 55 and 70% inhibition of the lysosome-bound enzymes was obtained.

A similar pattern of inhibition was observed when the neuraminidase assay was conducted in 0.25 M sucrose at pH 5.0 in order to minimize damage to the lysosomal membrane that could result from hypotonicity and low pH.

The effect of Triton X-100 on the neuraminidase activity of rat liver lysosomes, prepared and assayed as previously described[4], was also studied. In contrast with mammary glands, the activity of the lysosomal neuraminidase of rat liver increased upon addition of Triton X-100. The optimum concentration of detergent was 0.045% (v/v), which caused a 35% stimulation. Higher concentrations resulted in a gradual loss of this stimulation. At 0.3% (v/v) of Triton X-100 a 12% inhibition of the initial activity was observed. Similar results were obtained by HORVAT AND TOUSTER[18] who conducted studies in isotonic sucrose instead of aqueous suspension.

Effect of deoxycholate. Sodium deoxycholate is deleterious to both the soluble and lysosome-bound neuraminidases. Increasing concentrations of this salt resulted in proportional losses of enzyme activity. About 28% of the soluble and 50% of the lysosome-bound enzymes was inhibited at a final concentration of 0.4% sodium deoxycholate.

It is interesting to note that whereas most lysosomal enzymes are activated by Triton X-100 and by sodium deoxycholate, neuraminidase from rat mammary glands shows inhibition. Dimethylsulfoxide, which has been shown to increase acid phosphatase activity in aqueous suspensions of liver lysosomes[19], also failed to activate or solubilize the lysosomal neuraminidases at concentrations between 5 and 25% (v/v).

TABLE III

HYDROLYSIS OF VARIOUS SUBSTRATES BY THE NEURAMINIDASES FROM RAT MAMMARY GLANDS

Neuraminidase activity was measured with equivalent amounts of each substrate (400 nmoles of bound sialic acid). The soluble neuraminidase was the supernatant fraction from an aqueous homogenate of rat mammary glands (2.8 mg of protein per tube). The lysosomal preparations were aqueous suspensions of light and heavy lysosomes (0.8 and 1.4 mg of protein per tube, respectively).

Substrate	*Specific activity*		
	Soluble neuramini-nidase	*Lysosomal neuraminidases*	
		Light fraction	*Heavy fraction*
Neuramin–lactose	3.28	19.51	22.49
Neuramin–lactose sulfate	7.44	34.95	34.89
Brain gangliosides	0.72	7.70	9.17
Ovine submaxillary glycoprotein	0	5.13	4.77
Sialoglycopeptides	3.75	12.32	10.10

A moderate inhibition (5–20%) of the lysosome-bound enzyme was observed in these experiments.

Specificity. The rate of hydrolysis of various substrates by the soluble and the lysosomal neuraminidases of rat mammary glands is shown in Table III. With all three enzyme preparations, neuramin–lactose sulfate was hydrolyzed at a higher rate than neuramin–lactose. Although ovine submaxillary glycoprotein was not hydrolyzed by the soluble enzyme, the sialoglycopeptides isolated from pronase digests of this glycoprotein (7) were hydrolyzed at a rate similar to that obtained with neuramin–lactose. Mixed brain gangliosides were hydrolyzed by the soluble enzyme but at a rate proportionally much smaller than that obtained with the lysosome-bound neuraminidases.

TABLE IV

NEURAMINIDASE ACTIVITY IN THE SOLUBLE AND PARTICULATE FRACTIONS OF RAT MAMMARY GLANDS

All fractions were assayed for neuraminidase activity at pH 4.4 and 5.8 as described under *Methods*. The incubation mixtures were purified by ion-exchange chromatography[8] to eliminate interfering sucrose prior to the analysis of free *N*-acetylneuraminic acid.

Mammary gland fractions	*Total protein (mg)*	*Total enzyme*	
		pH 4.4 (units)	*pH 5.8 (units)*
Cytoplasmic extract in 0.25 M sucrose			
A. Supernatant fraction (105 000 × *g*, 1 h)	448	249	779
B. Sedimentable fraction (resuspended in 0.25 M sucrose)	138	480	217
Homogenate of Fraction B in 0.154 M KCl			
C. Supernatant fraction (105 000 × *g*, 1 h)	16	0	0
D. Sedimentable fraction (suspended in distilled water)	125	458	210

Intracellular localization of soluble neuraminidase

The soluble neuraminidase with pH optimum 5.8, which is present in the supernatant fraction of rat mammary gland homogenates, could either be a cytosol component or a particulate enzyme easily extracted by isotonic KCl (ref. 1) or by osmotic shock in aqueous homogenates.

In order to study this point, a rat mammary gland homogenate was prepared in 0.25 M sucrose (1:4, w/v). Nuclei and debris were removed by low-speed centrifugation, as described under *Methods*, and the cytoplasmic extract was then centrifuged for 1 h at 40000 rev./min (rotor No. 40). Neuraminidase activity was measured at pH 4.4 and 5.8 both in the supernatant fraction and in a portion of the particulate fraction resuspended in 0.25 M sucrose. As shown in Table IV, the supernatant fraction of the homogenate in 0.25 M sucrose had higher activity at pH 5.8 (soluble neuraminidase) while the particulate fraction, as expected, had higher activity at pH 4.4 (lysosomal neuraminidase). The remaining particulate fraction was homogenized in 0.154 M KCl and centrifuged at 40000 rev./min for 1 h. No soluble neuraminidase activity was extracted by the isotonic KCl while most of the lysosomal neuraminidase (pH optimum 4.4) was recovered in the aqueous suspension of the particulate fraction. Similarly, in experiments described above, treatment of lysosome-rich fractions with distilled water (Table II) solubilized only small amounts of neuraminidase which showed higher activity at pH 4.4 than at pH 5.8. These results indicate that the soluble neuraminidase with pH optimum 5.8 is a component of the cytosol.

DISCUSSION

The presence of a soluble neuraminidase in mammalian organs was first established in lactating mammary glands, liver and brain of rats[1].

Lysosome-bound neuraminidase was recently found in rat liver[3,18,20,21], rat kidney[20] and Ehrlich ascites tumor cells[18].

In a recent study with rat liver[4], it was demonstrated that the soluble and the lysosome-bound neuraminidases are actually two different enzymes which differ in their intracellular localization, in substrate specificity and in a number of enzymic properties.

The results of the experimental work described in this communication indicate that mammary gland also contains both a soluble neuraminidase in the cytosol and a particulate neuraminidase which is strongly bound to the lysosomes.

Fractionation of the nuclei-free particulate fraction of rat mammary glands by flotation through a discontinuous gradient of sucrose led to the isolation of two lysosome-rich fractions which collected at the 1.06–1.155 density and at the 1.155–1.21 density interfaces, respectively (Fig. 1). About 15-fold purification of the lysosome-bound neuraminidase is usually obtained by this procedure. Ultrastructural studies of these fractions (Fig. 2) revealed, in addition to lysosomes, the presence of contaminating membranous structures which probably originate from the smooth and rough endoplasmic reticulum. More than 80% of the particulate neuraminidase was recovered in these two fractions, while the remaining activity appeared in suspension in the sucrose layer of density 1.21. Aryl sulfatase, β-glucuronidase and acid phosphatase were also assayed in these same fractions. As shown in Table I, the first two enzymes showed a pattern of distribution similar to that of neuraminidase.

The pattern of distribution of acid phosphatase, on the other hand, suggests extra-lysosomal localization of a significant amount of this enzyme. This is further supported by the fact that about 50% of the acid phosphatase activity present in the cytoplasmic extract, remains in the supernatant fraction after isolation of the particulate fraction used in these studies. Another interesting observation is that while the specific activity of neuraminidase in the lysosomal fractions of rat mammary gland (Table I) is about one-tenth of that found in rat liver lysosomes[4], the specific activity of the acid phosphatase in rat mammary gland lysosomes is only one-twentieth of that present in rat liver lysosomes. These ratios indicate that rat mammary gland lysosomes have a proportionally lower content of acid phosphatase. Low levels of acid phosphatase activity of rat mammary gland were recognized by GREENBAUM *et al.*[22] in 1960. The values found in this study using unfractionated cytoplasmic extracts (0.33 μmole of phosphate/min per g wet weight) compare closely with the values obtained by HELMINEN *et al.*[23] with whole homogenates. The extra-lysosomal localization of acid phosphatase, both in other subcellular fractions as well as in the retained milk[23] complicates the interpretation of previous work concerning the physiological changes in the level of activity of this enzyme in mammary glands during lactation and involution.

Although histochemical proofs of the localization of the particulate neuraminidase are not yet available, the similarity of the relative distribution of neuraminidase, aryl sulfatase and β-glucuronidase in the subcellular fractions of rat mammary glands coupled to the fact that histochemical assay of the aryl sulfatase showed that this enzyme is present in the electron-dense bodies and could not be detected in the contaminating endoplasmic reticulum, strongly indicates that the particulate neuraminidase of mammary gland belongs, in all probability, to the group of the acid lysosomal hydrolases.

Procedures known to impair the integrity of the lysosomal membrane such as hypotonic shock, freezing and thawing, addition of sodium deoxycholate or of Triton X-100, usually solublized 10–15% of the bound neuraminidase (Table II), indicating strong binding of this enzyme to the lysosomal membrane. The pH optimum of the neuraminidase activity present in lysosomal suspensions, was found to be 4.4 (Fig. 3) while the soluble neuraminidase found in the supernatant fraction of mammary gland homogenates in isotonic KCl (refs. 1,3), in distilled water (Fig. 3) or in 0.25 M sucrose (Table IV), had maximum activity at pH 5.8. These differences are not due to allotopy[24] since the small amounts of solubilized lysosomal neuraminidase also showed higher activity at pH 4.4 than at pH 5.8.

The experimental evidence presented here indicates that the soluble neuraminidase is a different enzyme located in the cytosol. The possibility that the soluble neuraminidase could be a loosely bound lysosomal enzyme easily released by isotonic KCl (ref. 11) or by hypotonic shock can be ruled out since treatment of the particulate fraction with isotonic KCl failed to extract any neuraminidase activity (Table IV) and treatment of the lysosome-rich fractions with distilled water extracted only small amounts of activity (Table II) which, as stated above, had higher activity at pH 4.4 than at pH 5.8.

The effect of several mono- and bivalent cations on the mammary neuraminidases resemble those previously observed with the hepatic enzyme[4]. Cu^{2+}, Zn^{2+} and Hg^{2+} inhibited the soluble neuraminidase, but did not affect the lysosome-bound

enzyme (Fig. 6). Conversely, Li^+, Na^+ and K^+ inhibited the lysosomal neuraminidase but they did not inhibit the soluble enzyme (Fig. 7). The selective inhibition of the soluble and of the lysosomal neuraminidase with Cu^{2+} or with Li^+, respectively, was also demonstrated using suspensions of lysosome-rich fractions in aqueous soluble enzyme (Fig. 8).

Triton X-100 also affects these two enzymes quite differently; while the soluble enzyme was slightly stimulated, the lysosomal enzyme was strongly inhibited (Fig. 9). This inhibition is probably due to a combined effect of Triton X-100 which was found to increase the lability of the solubilized lysosomal neuraminidase (See Table II, Expts. b, c and e), in addition to having a direct inhibitory effect on the membrane-bound enzyme.

The different response of the neuraminidase of rat liver lysosomes, which is stimulated by low concentrations of Triton X-100, is of considerable interest. This effect cannot be ascribed solely to a disruption of the integrity of the lysosomal membrane, in fact, these observations were also done under conditions known to cause extensive damage of the lysosomal membrane (*i.e.* in aqueous suspensions at pH 4.4).

A comparative study of the rate of hydrolysis of various substrates (Table III) showed that neuramin–lactose sulfate is the best substrate for the neuraminidases of rat mammary gland. It was also observed that while the lysosomal neuraminidase hydrolyzed all the substrates tested, the soluble enzyme failed to act on ovine submaxillary glycoprotein. However, sialoglycopeptides isolated from pronase digests of this glycoprotein[7] proved to be a good substrate for the soluble neuraminidase. Brain gangliosides were hydrolyzed by the soluble neuraminidase of rat mammary gland but at a rate proportionally much slower than that obtained with the lysosomal enzyme.

Several cases of bimodal distribution, resulting from unimodal distribution of two distinct homologous enzymes, have been listed by DE DUVE[25]. A case that closely resembles the picture of the neuraminidases of rat liver[4] and rat mammary glands was recently described by PRICE AND DANCE[26] who found in rat kidney two different β-galactosidases, a soluble enzyme with pH optimum 5.5 and a lysosome-bound enzyme with pH optimum 3.7.

It must be pointed out that although most of the particulate neuraminidase has been found distributed in two fractions of different density, all the properties studied (pH optimum, K_m, specificity, effect of cations and detergents) failed to show any difference between the neuraminidases associated with these two lysosome-rich fractions. The polymorphism of lysosomes as well as their physical and chemical heterogeneity have been discussed by DE DUVE[27]. In addition to the well known variation in the properties of the lysosomes from one cell type to another, there is also some evidence for variations within a uniform population of mammalian cells in tissue culture[28]. Thus, the heterogeneity of the mammary lysosomes observed in these studies probably reflects not only differences in the functional state of these organelles but also the presence of lysosomes derived from diverse types of cells present in the glandular tissue.

The overall picture of the neuraminidases of rat mammary glands resembles that previously described in rat liver[4]. However, the marked contrast observed in the effect of Triton X-100 on the lysosomal neuraminidases from liver and mammary

gland suggests the existence of organ differences. Further comparative studies should be conducted before attempting an evaluation of the possible metabolic implications of organ differences of the mammalian neuraminidases.

Since the levels of activity of the soluble neuraminidase are known to undergo changes during lactation[3], a comparative study of the soluble and lysosomal neuraminidases of rat mammary glands during pregnancy, lactation and involution would probably give useful information concerning the metabolic role, interrelationship and regulation of these enzymes.

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REFERENCES

1 R. CARUBELLI, R. E. TRUCCO AND R. CAPUTTO, *Biochim. Biophys. Acta*, 60 (1962) 196.
2 G. L. ADA, P. E. LIND AND W. G. LAVER, *J. Gen. Microbiol.*, 32 (1963) 225.
3 B. H. TAHA AND R. CARUBELLI, *Arch. Biochem. Biophys.*, 119 (1967) 55.
4 D. R. P. TULSIANI AND R. CARUBELLI, *J. Biol. Chem.*, 245 (1970) 1821.
5 D. R. P. TULSIANI AND R. CARUBELLI, *Federation Proc.*, 29 (1970) 3034.
6 H. U. CHOI AND R. CARUBELLI, *Biochemistry*, 7 (1968) 4423.
7 R. CARUBELLI, V. P. BHAVANANDAN AND A. GOTTSCHALK, *Biochim. Biophys. Acta*, 101 (1965) 67.
8 L. W. MAYRON AND Z. A. TOKES, *Biochim. Biophys. Acta*, 45 (1960) 601.
9 L. WARREN, *J. Biol. Chem.*, 234 (1959) 1971.
10 A. B. ROY, *Biochem. J.*, 53 (1953) 12.
11 R. GIANETTO AND C. DE DUVE, *Biochem. J.*, 59 (1955) 433.
12 J. BERTHET AND C. DE DUVE, *Biochem. J.*, 50 (1951) 174.
13 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
14 A. TROUET, *Arch. Intern. Physiol. Biochem.*, 72 (1964) 698.
15 S. GOLDFISCHER, *J. Histochem. Cytochem.*, 13 (1965) 520.
16 J. T. DINGLE AND A. J. BARRETT, *Proc. Roy. Soc. London, Ser. B*, 173 (1969) 85.
17 C. S. HANES, *Biochem. J.*, 26 (1932) 1406.
18 A. HORVAT AND O. TOUSTER, *J. Biol. Chem.*, 243 (1968) 4380.
19 D. W. MISCH AND M. S. MISCH, *Proc. Natl. Acad. Sci. U.S.*, 58 (1967) 2462.
20 S. MAHADEVAN, J. C. NDUAGUBA AND A. L. TAPPEL, *J. Biol. Chem.*, 242 (1967) 4409.
21 N. N. ARONSON AND C. DE DUVE, *J. Biol. Chem.*, 243 (1968) 4564.
22 A. L. GREENBAUM, T. F. SLATER AND D. Y. WANG, *Nature*, 188 (1960) 318.
23 H. J. HELMINEN, J. L. E. ERICSSON AND S. ORRENIUS, *J. Ultrastruct. Res.*, 25 (1968) 240.
24 E. RACKER, *Federation Proc.*, 26 (1967) 1335.
25 C. DE DUVE, *J. Theoret. Biol.*, 6 (1964) 33.
26 R. G. PRICE AND N. DANCE, *Biochem. J.*, 105 (1967) 877.
27 C. DE DUVE, *Ciba Foundation Symp. on Lysosomes, London, 1963*, Little, Brown and Company, Boston, 1963, p. 1.
28 L. GORDIS AND H. M. NITOWSKY, *Exp. Cell Res.*, 38 (1965) 556.