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SOLUBLE AND LYSOSOMAL NEURAMINIDASES IN THE LIVER OF DEVELOPING CHICKS

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SUMMARY

1. Chick liver contains two neuraminidases (*N*-acetylneuraminate glycohydrolase, EC 3.2.1.18), one strongly bound to the lysosomes and another that occurs in soluble form in the cytosol.

2. The pH optima are 4.4 for the soluble and 3.8 for the lysosomal neuraminidase. The K_m for neuramin-lactose is $6.2 \cdot 10^{-4}$ M with both enzyme preparations. Cu^{2+} and Hg^{2+} inhibit the soluble but not the lysosomal neuraminidase. Preincubation at 37 °C results in decreased activity for the soluble enzyme and increased activity for the lysosomal preparation.

3. Both neuraminidases are fully active during the last week of embryonic life. The lysosomal enzyme remains fairly constant during pre- and postnatal development, while the soluble neuraminidase undergoes age-related changes, with a peak of maximum activity occurring 8 days after hatching.

INTRODUCTION

Neuraminidase (*N*-acetylneuraminate glycohydrolase, EC 3.2.1.18) activity in vertebrate cells was first reported by Ada and Lind[1] who found this enzyme in the chorioallantois of the chick embryo. Examination of unfractionated extracts prepared from various tissues of developing embryos and from one week old chicks showed the presence of this enzyme in all organs investigated[2].

This report deals with studies conducted on the neuraminidases of chick liver. The results obtained indicate the presence of a soluble and a lysosome-bound neuraminidase. The properties of these two homologous enzymes and their levels during postnatal and late prenatal development are described. The differences and similarities between the avian and mammalian neuraminidases are discussed.

Abbreviation: NANA, *N*-acetylneuraminic acid.

EXPERIMENTAL PROCEDURE

Materials

Neuramin-lactose and neuramin-lactose sulfate (potassium salts) were prepared as previously described[3]. Ovine submaxillary glycoprotein was a gift from Dr Alfred Gottschalk, Max Planck Institut, Tübingen, West Germany. Sialoglycopeptides were isolated from pronase digests of ovine submaxillary glycoprotein[4]. Crystalline synthetic *N*-acetylneuraminic acid (NANA) (Type IV), bovine brain gangliosides (Type III), β-glycerophosphate, *p*-nitrocatechol and *p*-nitrocatechol sulfate were products of Sigma, St. Louis, Mo. Fetuin (Grade B) from fetal calf serum, was purchased from Calbiochem, Los Angeles, Calif. All other chemicals utilized were of the highest purity commercially available.

White leghorn chicks of both sexes, obtained from a local hatchery, were used for this work. For studies of prenatal development, the embryos were removed from eggs incubated for the appropriate length of time and chilled briefly in ice-cold distilled water prior to sacrifice. For studies of postnatal development, the chicks were fed *ad libitum* on a commercial diet (All-in-one, obtained from Anderson Clayton and Co., Texas) and tap water. The animals were decapitated and the livers were quickly excised and chilled in ice-cold homogenizing solution. Fat, connective tissue, blood clots and gall bladder were carefully removed. The liver was then blotted, weighed, minced finely with sharp scissors, and homogenized as described below. An International Refrigerated centrifuge Model PR-2 was utilized for low speed centrifugation while a Spinco Model L Ultracentrifuge with rotors No. 30 and 40 was used for high speed centrifugation. All operations were conducted at 0–4 °C unless stated otherwise.

Methods

Preparation of the liver tissue fractions. Tissue homogenates (1:4, w/v) were prepared both in 0.154 M KCl and in 0.25 M sucrose–1 mM disodium EDTA. A glass homogenizer fitted with a mechanically driven teflon pestle (Arthur H. Thomas Co., Philadelphia) rotating at 1000 rev./min was utilized for this operation. After two up and down passes, the homogenates in 0.154 M KCl were centrifuged for 1 h at 105 000 $\times$ *g* and the clear supernate was decanted and used for the assay of the soluble neuraminidase. The homogenates in sucrose, obtained after a single up and down pass, were centrifuged for 10 min at 800 $\times$ *g*. The supernatant fraction was carefully decanted and the sediment was rehomogenized and centrifuged as above. The supernates were pooled and this cytoplasmic extract was then centrifuged for 1 h at 105 000 $\times$ *g*. The supernatant was poured off and excess sucrose was removed from the wall of the tubes and from the surface of the pellets by a quick rinse with ice-cold distilled water, taking special precautions not to disturb the sediment. The pellet, suspended in 4 ml of glass distilled water/g of initial wet weight of tissue, was then utilized for the assay of the particulate neuraminidase.

Enzyme assays. The enzyme preparations utilized for the neuraminidase assays consisted of 9 vol. of soluble or particulate liver fractions and 1 vol. of 1 M sodium acetate–acetic acid buffer. To 150 μl of enzyme preparation 50 μl of an aqueous solution of substrate (200 nmoles of neuramin-lactose) was added, and the mixture was incubated for 3 h at 37 °C. The pH of the reaction mixture was 3.8 for the particulate enzyme and 4.4 for the soluble enzyme.

The enzymic reaction was stopped by adding 0.1 ml of periodate reagent (5) and the free sialic acid was then determined by the thiobarbituric acid method of Warren as modified by Yeh *et al.*[6]. Enzyme and substrate controls were incubated concurrently and the corresponding readings were subtracted from that obtained with the complete system. Acid phosphatase (EC 3.1.3.2) was assayed at pH 5.0 with β-glycerophosphate as the substrate, according to Berthet and De Duve[7]. Arylsulfatase (EC 3.1.6.1) was assayed at pH 5.0 according to the method of Roy[8] using *p*-nitrocatechol sulfate as the substrate. One unit of neuraminidase activity is defined as the amount of enzyme that causes the release of 1 nmole of NANA per h under the conditions of the assay. The unit of enzyme activity for acid phosphatase and arylsulfatase is 1000 times larger, *i.e.*, the amount of enzyme that causes the hydrolysis of 1 μmole of substrate per h. Specific activities are expressed as units of enzyme activity per mg of protein.

RESULTS

Intracellular localization of chick liver neuraminidases

Tissue homogenates were prepared in 0.25 M sucrose–1 mM disodium EDTA and the nuclear fraction was separated from the cytoplasmic extract as described under *Methods*. Fractionation of the cytoplasmic extract by differential centrifugation yielded three successive particulate fractions: mitochondrial fraction (3500 × *g* for 10 min), lysosomal fraction (20 000 × *g* for 10 min) and microsomal fraction (105 000 × *g* for 60 min). All pellets were then suspended in homogenizing solution (1 ml per g of initial fresh tissue, except for the microsomal fraction which was suspended in 0.5 ml per g of initial wet weight of liver). Neuraminidase activity was assayed in all particulate fractions at pH 3.8 and in the final supernatant fraction at pH 4.4. The incubation was conducted as described under *Methods*, but because of the interference of sucrose with the thiobarbituric acid assay[9] the incubation mixtures were purified by ion-exchange chromatography[10] prior to the analysis of free NANA. Acid phosphatase and arylsulfatase were also assayed in all fractions. The enzymic activities and protein content of these fractions are shown in Table I. All three hydrolases exhibited the highest specific activity in the lysosome-rich frac-

TABLE I

INTRACELLULAR LOCALIZATION OF ACID HYDROLASES IN CHICK LIVER

Chick liver homogenates in 0.25 M sucrose–1 mM disodium EDTA were fractionated by differential centrifugation and the enzyme activities and protein content were measured in the isolated fractions as described in the text. The results represent the average of 3 experiments conducted with 20-day-old chicks.

Fraction	*Protein*	*Neuraminidase*		*Acid phosphatase*		*Arylsulfatase*	
	(mg/g liver)	*Activity (units/g liver)*	*Spec. act. (units/mg protein)*	*Activity (units/g liver)*	*Spec. act. (units/mg protein)*	*Activity (units/g liver)*	*Spec. act. (units/mg protein)*
Nuclear	30.5	44.2	1.45	5.68	0.19	1.60	0.05
Mitochondrial	35.6	42.6	1.20	3.78	0.11	1.39	0.04
Lysosomal	8.2	37.0	4.51	2.81	0.34	0.82	0.10
Microsomal	16.4	24.6	1.50	0.94	0.06	0.50	0.03
Supernatant	50.1	107.8	2.15	6.35	0.13	1.84	0.04

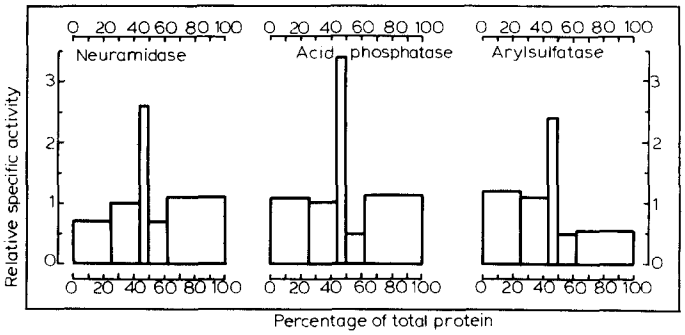


Fig. 1. Intracellular distribution of neuraminidase, acid phosphatase and arylsulfatase in chick liver. Ordinate: relative specific activity (percentage of total activity/percentage of total protein) of fractions; abcissa: relative protein content of fractions. Each pattern shows from left to right the values obtained for the nuclear, mitochondrial, lysosomal, microsomal and supernatant fraction respectively.

tion. The lysosomal nature of the particle-bound form of these three enzymes is also evident from the Relative Specific Activity profiles shown in Fig. 1.

Effect of sucrose on the activity of the soluble neuraminidase

Since initial experiments indicated that the soluble neuraminidase activity from livers homogenized in isotonic KCl was about 50% higher than that observed in the soluble fraction of similar homogenates in sucrose solution, a study of the effect of sucrose on the enzyme activity was conducted.

As shown in Fig. 2, (open circles) an increase in the molarity of the sucrose solution used in the preparation of the homogenate resulted in a gradual decline of the specific activity found in the 105 000 $\times$ *g*, 1 h supernate. Since the protein content remained fairly constant in these preparations, this appeared to be a direct effect of sucrose on the enzyme activity. This point was confirmed by assaying the neuraminidase activity of the soluble fraction obtained from a homogenate in distilled water, to which increasing amounts of sucrose were added. As shown in Fig. 2 (closed circles) a similar loss of activity was observed with increasing concentrations of sucrose in the incubation mixture.

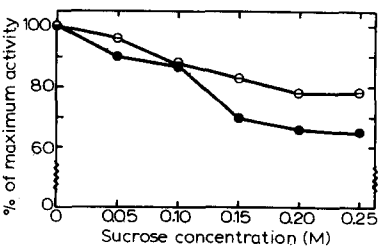
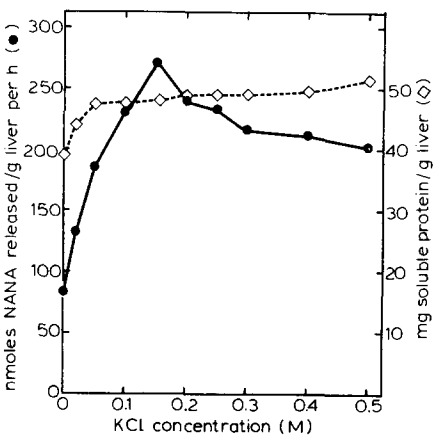


Fig. 2. Effect of sucrose on the activity of soluble neuraminidase. The activities obtained in the supernatant fractions of homogenates prepared with sucrose solutions of increasing molarities are shown by open circles. The amount of protein in mg per g of tissue extracted under these conditions was 48.4 $\pm$ 1.4 (mean $\pm$ S.D.). The decrease in activity observed upon addition of sucrose to the soluble fraction of an aqueous homogenate (1:2, w/v) is shown by the closed circles (final dilution of initial fresh tissue 1:4, w/v). The protein content of the soluble fraction used in the latter experiment was 46.2 mg/g fresh tissue. All chicks utilized were 8 days old.

Effect of KCl on the activity of the soluble neuraminidase

Since the specific activities in the soluble fractions of aqueous homogenates were considerably lower than those observed in the soluble fraction of similar homogenates prepared in isotonic KCl, the possible effect of this salt on the soluble neuraminidase was also studied. As shown in Fig. 3, the highest activity per g of fresh tissue was obtained in the soluble fraction of homogenates prepared in 0.154 M KCl. Although the increase in enzymic activity of the soluble fraction was accompanied by a slight increase in the protein concentration, the specific activity obtained with water is only 37% of that obtained with 0.154 M KCl. In view of the fact that the activity decreased in homogenates prepared with solutions of KCl of concentrations above 0.154 M, it was necessary to establish whether an optimum concentration of KCl is



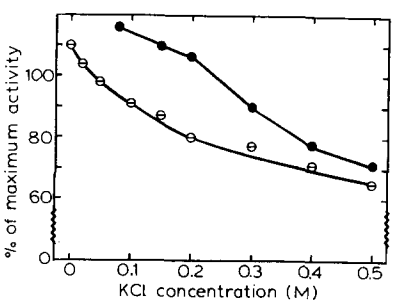


Fig. 3. Neuraminidase activity (●) and protein content (◇) of chick liver homogenates prepared with solutions of KCl of increasing concentrations. The experiments were conducted as described under *Methods*, except for the homogenizing solutions which were of the molarities shown in the graph.

Fig. 4. Effect of KCl concentration on soluble neuraminidase activity. Homogenates (1:2, w/v) were prepared in glass distilled water (open circles) or in 0.154 M KCl (closed circles) and the (105 000 × *g*, 1 h) supernate was isolated. The soluble fraction was then mixed with distilled water or with solutions of KCl of increasing molarity (final dilution of initial fresh tissue 1:4, w/v) and the neuraminidase assay was performed as described under *Methods*.

required for maximum activity or if the activity curve shown in Fig. 3 represents the balance between two opposing phenomena, *i.e.*, an increase in enzyme extraction with maximum at 0.154 M, on one hand, and an increase in enzyme inhibition by increasing KCl concentrations on the other hand. The latter turned out to be the case as shown by the results of the experiments depicted in Fig. 4. When the soluble fraction of an aqueous liver homogenate (1:2, w/v) was mixed with KCl solutions of increasing concentrations (final dilution of initial fresh tissue 1:4, w/v), a progressive loss of activity was observed with increasing concentrations of KCl in the incubation mixture (Fig. 4, open circles). In another experiment, a homogenate was prepared in 0.154 M KCl (1:2, w/v), instead of water, and the soluble fraction was diluted with water or mixed with KCl solutions of increasing concentrations. As shown in Fig. 4 (closed circles) a similar pattern of inhibition was observed with increasing concentra-

tions of KCl, while some activation occurred upon dilution of this preparation with glass distilled water.

The sedimentable fraction (105 000 × *g*, 1 h), of chick liver homogenates in isotonic KCl retained more than 25% of the total activity firmly bound to the particulate fraction. Two successive treatments of this pellet with fresh isotonic KCl failed to extract any significant amount of neuraminidase activity and the specific activity of this particulate fraction remained constant throughout the experiment (Table II).

TABLE II

REPEATED EXTRACTION OF CHICK LIVER PARTICULATE FRACTION WITH ISOTONIC KCl SOLUTION

The cytoplasmic extract obtained from a chick liver homogenate in 0.154 M KCl (1:4, w/v) was centrifuged for 1 h at 105 000 × *g* and the pellet was resuspended in 0.154 M KCl to its initial volume. The centrifugation and KCl treatment was then repeated.

Fraction	*Total protein/g liver (mg)*	*Recovery (%)*	*Total enzyme/g liver (units)*	*Recovery (%)*	*Spec. act.*
Cytoplasmic extract	104.9	100.0	251.9	100.0	2.4
Supernatant fraction I	56.8	54.1	189.1	75.1	3.3
Particulate fraction I	43.8	41.8	72.1	28.6	1.6
Supernatant fraction II	2.8	2.7	4.7	1.9	0.7
Particulate fraction II	40.9	39.0	65.0	25.9	1.6
Supernatant fraction III	1.2	1.1	0.0	0.0	0.0
Particulate fraction III	36.9	35.2	58.6	23.3	1.6

Comparative study of soluble and lysosomal chick liver neuraminidases

Kinetic studies. The pH *versus* activity curve for soluble neuraminidase, showed a broad peak with highest activity at pH values between 4.2 and 4.4 (Fig. 5). The particulate enzyme, on the other hand, showed a narrower peak with a pH optimum of 3.8; a rather sharp drop of activity occurred at pH values above 4.0. A linear rate of hydrolysis for up to 3 h and a linear relationship between protein concentration and NANA released were observed with both enzyme preparations. The K_m values for neuramin-lactose, calculated from the $[S]/v$ *versus* $[S]$ plots[11] are $6.18 \cdot 10^{-4}$ M and $6.24 \cdot 10^{-4}$ M for the soluble and particulate neuraminidases respectively (Fig. 6).

Effect of bivalent cations. The effect of several bivalent cations was studied by the addition of the respective chlorides to the incubation mixtures. At 1 mM concentration (see Table III) Cu^{2+} and Hg^{2+} caused strong inhibition of the soluble neuraminidase, while the particulate enzyme showed a very small loss of activity. Mg^{2+}, on the other hand, did not affect the soluble enzyme but caused stimulation of the particulate neuraminidase. Ca^{2+} and Zn^{2+} had very little effect on the enzyme activity of either preparation.

Stability. No loss of neuraminidase activity was detected when the soluble enzyme was stored at 0 °C for 24 h; after 3 days, 15–20% losses were observed. The particulate enzyme, on the other hand, lost 20–25% of its initial activity upon storage at 0 °C for 24 h.

Effect of preincubation. Considerable changes of activity were observed when these enzymes were incubated at 37 °C prior to assay (Fig. 7). The soluble enzyme experienced a progressive loss of activity, which amounted to about 60% of the initial

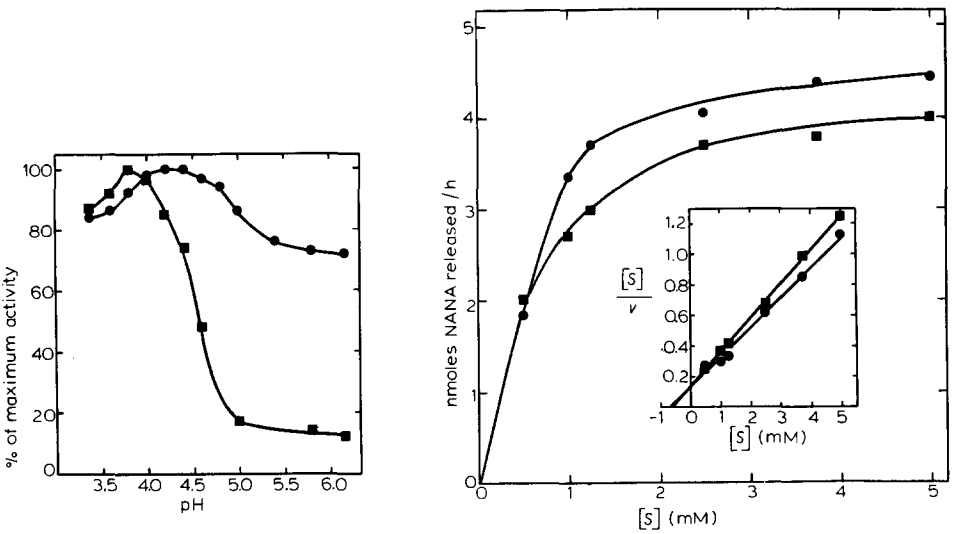


Fig. 5. pH curves of chick liver neuraminidases. The circles correspond to the soluble enzyme (1.97 mg of protein per sample), the squares correspond to the particulate enzyme (1.89 mg of protein per sample). The pH values shown were obtained with sodium acetate–acetic acid buffers (75 mM final concentration).

Fig. 6. Effect of neuramin-lactose concentration on the rate of hydrolysis. The assays were conducted with the soluble (circles) and the particulate (squares) fractions of livers obtained from 24-day-old chicks. The assays were conducted as described under *Methods*, except for the substrate concentration which varied as shown. The amount of protein per tube was 1.75 mg for the soluble fraction and 1.82 mg for the particulate enzyme.

TABLE III

EFFECT OF BIVALENT CATIONS ON CHICK LIVER NEURAMINIDASES

The cations were added to the incubation mixtures as their chlorides (1 mM final concentration) and the assay was conducted as described under *Methods*.

Cations	*Percent of initial activity*	
	Soluble neuraminidase	*Particulate neuraminidase*
Cu^{2+}	11	87
Hg^{2+}	3	92
Mg^{2+}	100	122
Ca^{2+}	103	99
Zn^{2+}	105	110

activity at the end of 2 h. Conversely, the particulate enzyme, subjected to the same treatment, experienced between 45 and 90% stimulation.

Specificity. A comparative study of the rates of hydrolysis of various substrates by the soluble and particulate neuraminidases of chick liver is shown in Table IV. Low molecular weight substrates such as neuramin-lactose, neuramin-lactose sulfate and sialoglycopeptides from ovine submaxillary glycoprotein were hydrolyzed more efficiently than the macromolecular substrates tested. Intact salivary mucin and gangliosides were poor substrates for both preparations, and the particulate enzyme gave

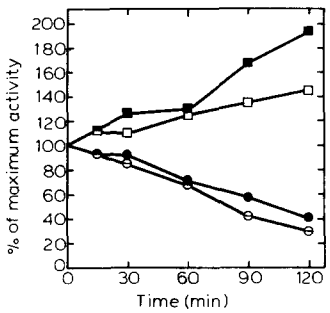


Fig. 7. Effect of preincubation on chick liver neuraminidases. The enzyme preparations were incubated in a water bath at 37 °C and chilled in an ice-bath at the end of the various periods shown. Substrate was then added and all enzyme assays were performed simultaneously as described under *Methods*. Circles represent soluble enzyme (closed, 10 days old; open, 30 days old); squares represent particulate enzyme (closed, 10 days old; open, 30 days old).

TABLE IV

HYDROLYSIS OF VARIOUS SUBSTRATES BY THE SOLUBLE AND PARTICULATE NEURAMINIDASES FROM CHICK LIVER

Neuraminidase activity was assayed with equivalent amounts of each substrate (200 nmoles of bound *N*-acetylneuraminic acid) as described under *Methods*. The liver tissue was obtained from 12-day-old chicks.

Substrate	*Neuraminidase activity (nmoles NANA released/h per mg protein)*	
	Soluble	*Particulate*
Neuramin-lactose	4.5	2.4
Neuramin-lactose sulfate	6.9	4.6
Sialoglycopeptides	2.9	5.0
Ovine submaxillary glycoprotein	0.2	0.4
Mixed brain gangliosides	0.4	0.9
Fetuin	0.0	0.0

higher specific activities than the soluble preparation. Fetuin was not attacked by these enzyme preparations.

Developmental changes of chick liver neuraminidases

Soluble neuraminidase. This enzyme was fully active during the last week of embryonic life. After hatching, a gradual increase in enzyme activity was observed during the first week, followed by a gradual decrease in specific activity from the second through the sixth week of life (Fig. 8). Since the protein content of this fraction remained fairly constant at 58.05 ± 5.78 (mean ±S.D.) per g of fresh tissue, a plot of activity per g of tissue shows an identical profile.

The possibility of a neuraminidase inhibitor, which would explain the age-dependent drop in the activity of the soluble enzyme (Fig. 8), was ruled out after experiments conducted with mixtures of the soluble fraction from livers of 8- and 34-day-old chicks showed quantitative recoveries of enzyme activity. Similar results were obtained with mixtures of the particulate fractions from the same organs.

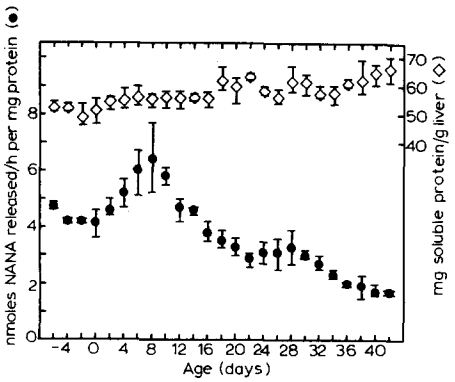


Fig. 8. Neuraminidase activity (●) and protein content (◇) of the soluble fraction of chick liver during development. The points represent the mean and the vertical bars indicate the range of values obtained with 3–4 different enzyme preparations.

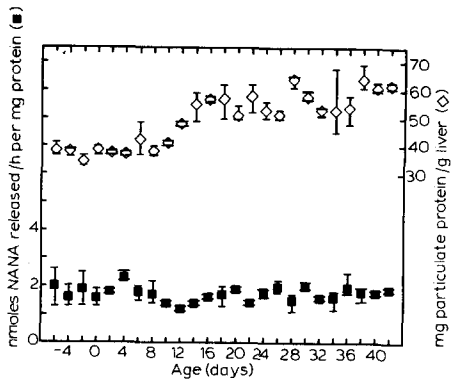


Fig. 9. Neuraminidase activity (■) and protein content (◇) of the particulate fraction of chick liver during development. The points represent the mean and the vertical bars indicate the range of values obtained with 3–4 different enzyme preparations.

Particulate neuraminidase. The specific activity in the liver of chick embryos was similar to that observed in 40-day-old chicks (Fig. 9), and no definite pattern of changes was observed through the age period studied. Since the protein content of this fraction expressed in mg per g of fresh liver increases with age, the liver of 40-day-old chicks contains about twice as much particulate neuraminidase as the liver from chick embryo, when the enzyme activity is expressed per g of fresh tissue.

DISCUSSION

The high specific activity of the neuraminidase present in the lysosome-rich fraction (Table I) and the similarities between the relative specific activity profiles of this enzyme and those of the lysosomal markers acid phosphatase, and arylsulfatase (Fig. 1) indicate the lysosomal nature of the particle-bound neuraminidase of chick liver. On the other hand, the presence of neuraminidase activity in the high speed supernate obtained from homogenates prepared in distilled water, isotonic KCl and 0.25 M sucrose, indicate the presence of a soluble neuraminidase located in the cytosol of these cells. The strong binding of the lysosomal enzyme, which cannot be extracted by repeated treatment of the particulate fraction with isotonic KCl, (Table II) and the marked differences between the enzymic properties of the soluble and particulate preparations further supports the presence of two homologous enzymes, a situation similar to that previously observed in rat liver[12].

The pH optima for the soluble and for the lysosomal neuraminidases of the chick liver are considerably lower than those found for the corresponding enzymes of rat liver[12]. The measurements of neuraminidase activity performed by Cook and Ada[2] in unfractionated cytoplasmic extracts of various chick tissues, including liver, were conducted at pH 5.8. Since these authors used a different strain of chick and their tissue preparations were subjected to dialysis prior to assay, it is not clear at this time whether this elevated pH optimum reflects genetic differences or if the prolonged

dialysis, which is known to cause inhibition of the rat lysosomal neuraminidase[9] caused alterations in the properties of their enzyme preparation. The fact remains that under our experimental conditions, measurements at pH 5.8 would detect only 73% of the soluble and 14% of the lysosomal neuraminidases of chick liver (Fig. 5).

The K_m values found for neuramin-lactose are identical for the soluble and particulate neuraminidases of chick liver. It must be pointed out that while the K_m obtained with the particulate enzyme of rat and chick livers are very similar, the K_m for the soluble enzyme of chick liver is one fourth of that obtained with the rat liver enzyme[13].

The results obtained upon addition of Cu^{2+} or Hg^{2+} to the neuraminidases of chick liver (Table III) are similar to those previously observed with the neuraminidases of liver, brain and mammary glands of rats[12–14]. However, the lack of effect of Zn^{2+} was unexpected since this cation caused clear inhibition of the soluble mammalian neuraminidase. The inhibition of the soluble chick neuraminidase by KCl also points out an additional difference with the mammalian enzyme, since this salt was shown to inhibit the particulate but not the soluble mammalian neuraminidase[12–14].

The specificity studies (Table IV) showed poor activity of both chick liver neuraminidases toward high-molecular-weight substrates, while in the case of rat liver the particulate enzyme was quite effective with both low and high molecular weight substrates[13].

Although organ and species variations do occur[13], a trend common to all developmental patterns studied so far is that while the specific activity of the particle-bound neuraminidase remains fairly constant, the soluble neuraminidase undergoes age-related changes during late prenatal or early postnatal development.

The need for two homologous neuraminidases with different intracellular localization and different enzymic properties remains a puzzling and challenging question. Since sialic acid has been shown to play an important role in the regulation of the survival of glycoproteins in circulation[15], it is reasonable to assume that in addition to its well established catabolic function, the neuraminidases of vertebrate tissues might also play an important role in metabolic regulation. The binding of circulating glycoproteins to the plasma membrane of the parenchymal cells of the liver[16] requires the presence of sialic acid on the membrane and its absence from the glycoprotein molecule. As recently postulated by Pricer and Ashwell[16], the reversible binding and release of glycoproteins, which is mediated by the removal or insertion of sialyl groups, could perhaps be accomplished by the combined action of the cytoplasmic neuraminidase and the membrane-bound sialyltransferase respectively. If this were the case, the soluble neuraminidase could play an important role in the regulation of the biological activity of a number of sialoglycoproteins. On the other hand, since the uptake of some plasmatic glycoproteins requires the cleavage of only a small fraction of the sialic acid residues[17], further catabolic degradation of these macromolecules could then proceed by removal of the remaining sialic acid residues accomplished by the action of the lysosomal neuraminidase.

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