In this paper we wish to report on the susceptibility of eledoisin heterosequences towards hydrolysis by organ homogenates and aminopeptidases.

Materials and methods. The organ homogenates were prepared by a method published by NOBILI[8]. Aminopeptidase M (AP-M) was purchased from Röhm and Haas Co., Darmstadt. Pure leucine aminopeptidase (LAP) from bovine eye lenses was kindly supplied to us by Prof. HANSON, Halle (GDR). Peptide substrates were incubated with organ homogenates at 37 °C. The rate of cleavage was estimated by measuring the contraction of residual peptide in isolated guinea-pig ileum after dilution to the suitable concentration range of the organ bath. Incubation sample contains 0.3 ml peptide solution, 2.55 ml phosphate buffer, pH 7.4, and 0.15 ml homogenate from 40 mg moist organ. The peptide concentration was 2.4×10^{-7} *M*.

In the experiments with aminopeptidases (for results see Table) 350 mU AP-M or 1.5 mg LAP were used per µmol peptide for enzymatic hydrolysis of eledoisin heterosequences. Conditions were: 20 h, 37 °C, pH 8.9 for LAP and 7.7 for AP-M. Incubation was followed by determination of the amino acid released, using an automatic amino acid analyser.

Hydrolysis of heteropeptides by aminopeptidases

Peptide	% Recovery for			
	Lys	Ala	Ile	
Lys-Asn-Ala-Phe-Ile-Gly-Leu-Met-NH_2	76	70	73	LAP
Lys-Azasn-Ala – – – – – – – – – – I	78	1	1	LAP
Lys-N(Ac)-Gly-Ala – – – – – – – – – – I	7	2	5	AP-M
Ala-NHPhe(Ac)-Ile – – – – – – – – – I	—	73	3	AP-M
Ala-N(Ac)-Phe-Ile – – – – – – – – – – I	—	2	2	AP-M

Azasn = –NH–N(CH_2–$CONH_2$)CO–, N(Ac)—Gly = N^{α}-acetyl hydrazinoacetyl (–NH–N^{α}(Ac)–CH_2–CO), NHPhe = α-hydrazino-β-phenylpropionic acid.

Results and discussion. The following guinea-pig organs were used: liver, kidney, ileum, skeletal muscle, lungs, spleen, uterus, tube, seminal vesicle. While complete inactivation of 5-Asn-eledoisin-4-11 and analogues modified in position 7 and 9 occurs with most of the organ homogenates within 15–30 min, no loss in activity of 5-Azasn-eledoisin-4-11 during the same period of time was observed. Only liver homogenate shows a low rate of degradation with 50% cleavage in about 30 min.

Unmodified eledoisin sequences are cleaved completely by aminopeptidases; but in heterosequences with hydrazino carbonic acids or aza amino acids, only the amino acid residue just before the heteroconstituent is removed. In acetylated hydrazino peptides, the enzyme only eliminates a trace of the amino acid preceding the heteroconstituent. Such modification in cleavibility may certainly be attributed to deviation from the natural peptide structure.

In hydrazino peptides, the alterations are a chain elongation of about 1.2 Å and a torsion of about 60° for the side chain of the hydrazino acid or for the subsequent peptide chain, as judged by molecular models. Azapeptides contain a relatively rigid semicarbazide structure, caused by their urea-like mesomerism[5]. The consequence would be a considerable steric change in the azapeptide molecule. This assumption is supported by a significant alteration in circular dichroism spectra (1 positive maximum at 210 nm) and by the fact that also chymotrypsin and pronase show a retarded inactivation of 5-Azasn-eledoisin-4-11.

Zusammenfassung. Analoge des Eledoisin-Oktapeptides 4-11, die Hydrazid-Komponenten enthalten, sind durch Aminopeptidasen nur bis zur Heterobindung abbaubar. Sie werden wesentlich langsamer als die native Sequenz bzw. nicht durch Organhomogenate inaktiviert, wenn die Substitution im N-terminalen Bereich erfolgt. Mögliche sterische Veränderungen des Peptids durch eingebaute Fremdbausteine sind diskutiert.

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[8] M. B. NOBILI: Archs inter. Pharmacodyn. *158*, 187 (1965).

Phosphatases IX[1]. Differences in Sialic Acid Content of Rat Liver Alkaline Phosphatase Isoenzymes

The heterogeneity of alkaline phosphatase (orthophosphoric monoester phosphohydrolase, E.C. 3.1.3.1) within a single tissue, as demonstrated by gel electrophoresis of tissue extracts, has frequently been noted[2–4]. For rat liver 2 main isoenzymes have been found[5–7]. In our previous experiments, different response of the 2 isoenzymes to bile duct ligation was reported[5]. The present communication deals with the different response of the 2 isoenzymes to neuramidase treatment.

Material and methods. Adult male Wistar rats (approx. 200 g body wt.), fed a standard food diet and fresh water ad libitum, were killed by decapitation. The liver was perfused with ice-cold 0.25 *M* sucrose, rapidly removed, weighed and homogenized in cold 0.25 *M* sucrose with a Potter-Elvehjem homogenizer fitted with a Teflon-pestle. The nuclear and mitochondrial fractions were prepared according to APPELMANS et al.[8]. Both fractions were washed 3 times. Alkaline phosphatase was extracted from the homogenate and from the subcellular fractions[9]. *n*-Butanol was added to a final concentration of 20%, the mixture was warmed up to 37 °C and stirred for 15 min. The aqueous phase was obtained after centrifugation at 20,000 x *g* (4 °C) for 30 min, dialyzed overnight against 0.05 *M* sodium acetate and used for the experiments.

The preparations were incubated with neuraminidase at 37 °C overnight. 1 mg of neuraminidase from *Clostridium perfringens* (Sigma) was dissolved in 0.1 ml of 0.1 *M* sodium acetate, 0.15 *M* NaCl, 0.01 *M* $MgCl_2$ at pH 5.5.

To 0.8 of the alkaline phosphatase preparation, 0.22 ml of 0.2 *M* acetate buffer at pH 5.5 and 0.04 ml of the neuraminidase solution was added.

The liver alkaline phosphatase preparations were separated by agar gel electrophoresis[10]. Ionagar No. 2 (Oxoid, England) 1.5% in 0.04 *M* barbital buffer at pH 8.3 was used. The separation was done at 4 °C for 60 min at 30 V/cm and 5 mA/cm. The enzyme activity bands were visualized by incubation at 37 °C for 60 min in 10 ml of 0.05 *M* carbonate-bicarbonate buffer at pH 9.8, containing 5 mg of Naphthol As-BI phosphoric acid sodium salt (Sigma) and 3 mg of Echtblausalz BB (Hoechst).

Alkaline phosphatase activity was determined for 10 m*M* *p*-nitrophenyl phosphate (B.D.H., England) in 0.05 *M* carbonate-bicarbonate buffer at pH 10.0 containing 5×10^{-4} $MgCl_2$. *p*-Nitrophenol released in 30 min at 25 °C was measured by optical density at 405 nm[11].

Results. In the extracts of the whole rat liver homogenate, a fast and a slow anodic band and a cathodic band of alkaline phosphatase activity were found (Figure). The activity of the fast anodic band was very weak. In the nuclear fraction, only the fast anodic band, and in the extracts of mitochondria, only the cathodic one was demonstrated.

After the neuraminidase treatment, the originally anodic bands of alkaline phosphatase extracted from the whole homogenate and from the nuclear fraction were shown to be cathodic now. The electrophoretic mobility of the cathodic band in extracts of the whole homogenate and of mitochondria was not affected by the neuraminidase treatment, not even after a 3 day incubation (Figure).

After experimental bile flow obstruction in rats, the activity of the two fast anodic bands of the liver alkaline phosphatase is strongly increased and appears also in the serum[5, 12, 13]. The neuraminidase treatment of this isoenzyme of serum alkaline phosphatase after cholestasis resulted in the same change in electrophoretic mobility as observed for anodic isoenzyme in the normal rat liver (Figure). This is in agreement with the results found by KAPLAN and RIGHETTI[13]. The total activity of alkaline phosphatase was not changed after the neuraminidase treatment.

Discussion. The bulk of rat liver alkaline phosphatase activity is localized in the liver cell membranes[14]. As shown previously, this activity is represented by the anodic bands of the enzyme in agar gel electrophoresis[5]. These bands are interconvertible[5]. Their electrophoretic mobility is retarded by neuraminidase treatment. This is due to release of sialic acid residues from the enzyme molecule, as verified for placental, bone and also liver alkaline phosphatase[15].

The cathodic band of the liver alkaline phosphatase was found in the mitochondrial fraction, probably as a result of adsorption of the cytosol enzyme to mitochondria[6, 16]. This isoenzyme does not contain the sialic acid residues which could be split off by the neuraminidase treatment. The same characteristic is found for the intestinal isoenzyme of serum alkaline phosphatase[15].

Summarizing, the rat liver alkaline phosphatase is heterogenous, the 2 isoenzymes differ in their subcellular localization, electrophoretic mobilities, response to extrahepatic cholestasis[5] and their activation by Mg^{2+} and inhibition by KCN and deoxycholate[7]. Finally, they differ also in their sialic acid content.

Zusammenfassung. Die Heterogeneität der alkalischen Phosphatase in der Rattenleber wird beschrieben. Anodische und kathodische Isoenzyme unterscheiden sich unter anderem durch ihre Reaktion auf die Neuraminidase Behandlung.

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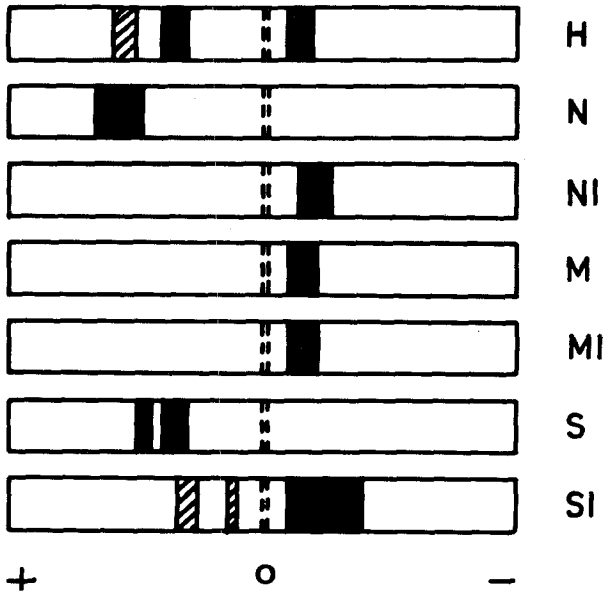
9 February 1973.



Influence of the incubation with neuraminidase on the electrophoretic mobilities of alkaline phosphatase of rat liver and serum. H, whole liver homogenate; N, nuclear fraction; M, mitochondrial fraction; S, serum of the rat with extrahepatic cholestasis. NI, MI and SI, fractions treated with neuraminidase. 0, origin.

[1] V. VOLEK, J. ŠTĚPÁN, E. BENEŠOVÁ and F. HEŘMANSKÝ, Sb. lék. in print (1973).
[2] D. W. Moss and E. J. KING, Biochem. J. *84*, 192 (1962).
[3] J. K. SMITH, R. E. EATON, L. G. WHITBY and D. W. Moss, Analyt. Biochem. *23*, 84 (1968).
[4] D. W. Moss, Ann. N.Y. Acad. Sci. *166*, 641 (1969).
[5] H. BÖRNIG, J. ŠTĚPÁN, A. HORN, R. GIERTLER, G. THIELE and B. VEČEREK, Hoppe-Seyler's Z. physiol. Chem. *348*, 1311 (1967).
[6] R. KASCHNITZ, J. PATSCH and M. PETERLIK, Eur. J. Biochem. *5*, 51 (1968).
[7] A. KANEKO, J. Biochem. *64*, 785 (1968).
[8] F. APPELMANS, R. WATTIAUX and C. DE DUVE, Biochem. J. *59*, 438 (1955).
[9] R. H. MORTON, Biochem. J. *57*, 595 (1954).
[10] J. ŠTĚPÁN and B. VEČEREK, Chemické Listy, in print (1973).
[11] L. KADLECOVÁ and J. ŠTĚPÁN, Experientia *28* 1284 (1972).
[12] J. ŠTĚPÁN, B. VEČEREK and K. KÁCL, Acta hepato-splenol. *15*, 331 (1968).
[13] M. M. KAPLAN and A. RIGHETTI, J. clin. Invest. *49*, 508 (1970).
[14] P. D. R. HOUSE and M. J. WEIDEMANN, Biochem. biophys. Res. Commun. *41*, 541 (1970).
[15] W. H. FISHMAN and N. K. GHOSH, Adv. clin. Chem. *10*, 255 (1967).
[16] P. M. STEVENSON and P. L. TAYLOR, FEBS Lett. *19*, 251 (1971).