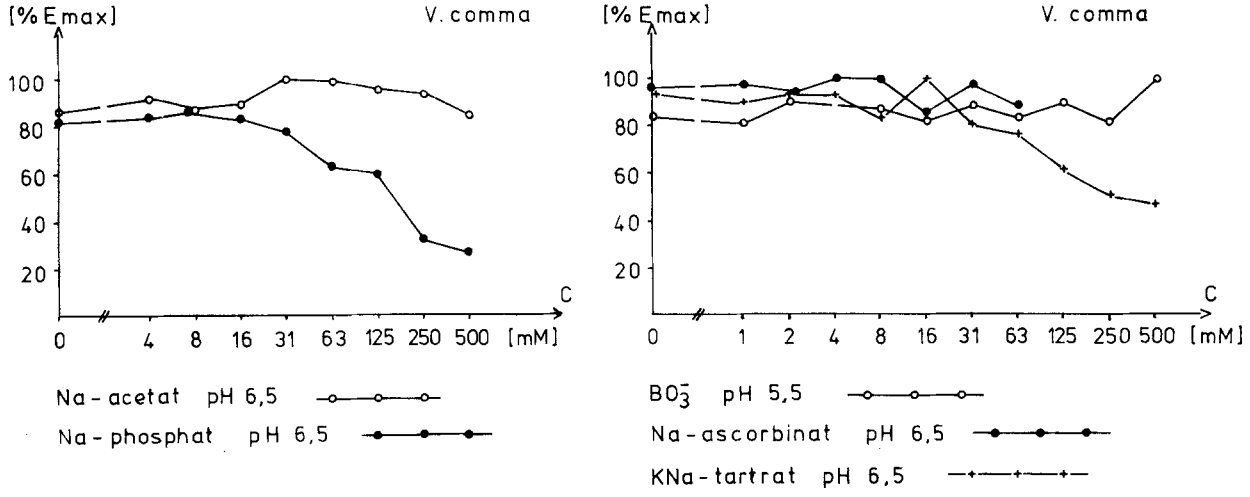

Fig. 2. Der Einfluss von Natrium-Acetat, -Phosphat, -Borat, -Ascorbinat und Kalium-Natrium-Tartrat auf die Aktivität der Cholera-Neuraminidase.

gradienten von Na-Pyruvat, Na-Citrat, Na-Oxalat und Na-EDTA. Ausserdem ist in Figur 2 der Einfluss von Acetat, Phosphat (linkes Bild), Borat, Ascorbinat und Tartrat (rechtes Bild) auf Cholera-Neuraminidase dargestellt.

Die Aktivität der Neuraminidasen wurde über enzymatisch frei gesetztes N-Acetylneuraminat mit Hilfe der Thiobarbiturat-Methode nach WARREN bestimmt[10,12]. Die Neuraminidasen von *Erysipelothrix insidiosa*, *Pasteurella multocida* und *Streptococcus viridans* wurden von Bakterien-Stämmen gewonnen, die aus klinischem Material stammten. Bei der Cholera-Neuraminidase handelte es sich um ein kommerzielles Produkt.

Aus den Ergebnissen der Figuren 1 und 2 geht hervor, dass die Anionen, die mit Calcium-Ionen unlösliche Salze oder Komplexverbindungen bilden, also Citrat, EDTA, Oxalat, Phosphat und Tartrat zu gleichsinnigen Hemmeffekten bei Cholera-Neuraminidase führen. Dagegen findet sich kein Einfluss der Ca··-bindenden Anionen bei den Neuraminidasen von *Erysipelothrix insidiosa* und *Streptococcus viridans*, bei der *Pasteurella multocida*-Neuraminidase ist der Effekt nur schwach ausgeprägt.

Aber ausserhalb der Reihe von Anionen mit hoher Ca-Ionen-Affinität, die nur bei Cholera-Neuraminidase voll wirksam waren, inhibiert auch das Pyruvat in den etwas höheren Konzentrationen von etwa 60–250 m*M* die Aktivität aller vier untersuchter Neuraminidasen unabhängig von ihrer Inaktivierbarkeit durch Calcium-Komplexbildner. Es dürfte sich hier um einen spezifischen Hemmeffekt handeln, der keine Beziehung zu der bei Cholera-Neuraminidase beobachteten Ionenaktivierbarkeit besitzt. Eine unspezifische Hemmwirkung lässt sich zumindest ausschliessen, denn weder Acetat noch Borat noch NaCl entfalten bei gleich hohen oder höheren molaren Konzentrationen derartige Hemmwirkungen. Auch Citrat scheint in ähnlicher Weise wie Pyruvat zu wirken, denn es zeigt im Gegensatz zu den übrigen Calcium-Komplex-bildenden Anionen auch eine Hemmwirkung bei *Streptococcus viridans*-Neuraminidase und in geringerem Mass auch bei *Erysipelothrix insidiosa*-Neuraminidase, die durch Ca-Ionen nicht aktiviert werden. Interessanterweise handelt es sich sowohl bei Pyruvat als auch bei Citrat um wichtige Stoffwechselprodukte des Citronensäure-Zyklus.

Das Problem der Hemmung von Neuraminidasen pathogener Mikroorganismen mag deshalb einige Bedeutung haben, weil sich vielleicht durch gezielte Steuerung des Stoffwechsels oder durch Applikation wirksamer Substanzen die Enzymaktivität senken und damit pathogene Wirkungen verringern lassen[13].

Summary. It was shown that neuraminidase of *Vibrio comma* is inactivated by Ca··-binding anions like citrate, EDTA, oxalate, phosphate or tartrate. There is, however, no inhibition of the newly described enzymes of *Erysipelothrix insidiosa* and *Streptococcus viridans*. Pyruvate and, to a lesser extent, also citrate inactivate all the neuraminidases investigated independently of their activation by Ca·· ions.

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17. Dezember 1974.

[12] L. WARREN, J. biol. Chem. *234*, 1971 (1959).
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Purification of N-Acetyl-Hexosaminidase from Boar Epididymis by Affinity Chromatography

Of the several mammalian tissues examined, the epididymis of the adult mammalian male provides the richest source of N-acetylhexosaminidase (β-2 acetamido-2 deoxy-D-glucopyranoside acetylaminodeoxyglucohydrolase, E.C. 3.2.1.30)[1,2]. From the observation, that the epididymis of the adult male contains about 10-fold the enzyme concentration of the infantile tissue, it can be derived that this glycosidase is under endocrine control.

Based on the findings by WEIL[3], that *p*-aminophenyl-N-acetyl-β-D-thioglucosaminid is a potent reversible inhibitor of N-acetylhexosaminidase from boar epididymis,

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we have developed an adsorbent with *p*-aminophenyl-N-acetyl-β-D-thioglucosaminide as ligand of paramount usefulness for affinity chromatography of the enzyme in order to facilitate purification of N-acetylhexosaminidase.

The ligand (*p*-aminophenyl-N-acetyl-β-D-thioglucosaminide) was a gift of Dr. WEIL and the synthesis was performed according to a published method for the synthesis of the corresponding oxygen analogue[4].

Acetochloroglucosamine (25 g, 68,5 mmoles), prepared according to LEABACK[4], and *p*-nitrothiophenol (14,25 g, 102.5 mmoles) were shaken for 10 min with 250 ml acetone; 1 *N* NaOH (102.5 ml) was then added. The solution was left for 16 h at 4 °C, the crystalline solid collected, washed with 0.02 *N* NaOH until the washings were colourless and dried to give *p*-nitrophenyl-2,3,4,6-tetraacetyl-β-D-thioglucosaminide (10–11 g, m.p. (dec.) 185–188 °C which can be used without further purification. The tetraacetate (1 g) in 50 ml methanol was reduced in a Parr apparatus (6 h, 40 mm pressure, in Parr) in the presence of 100 mg Pd/C (10%). The reaction mixture was centrifuged, the pellet washed with methanol/HCl (2% v/v 1.0 *M* HCl) and the methanol extracts dried in vacuo. The material was redissolved in methanol and deacetylated in the presence of 0.01 *M* methanolic sodium methoxide. The precipitated material was redissolved in 10 ml ice cold 0.1 *M* sodiumphosphat buffer pH 7.5 and added to a 1:1 suspension in dioxane of 100 ml of the N-hydroxysuccinimideester of succinylated aminoalkyl agarose derivative[5]. The filtered, substituted agarose was washed with methanol, dioxan and water. Before use, the resin was equilibrated with 0.1 *M* citrate phosphate buffer, pH 4.5.

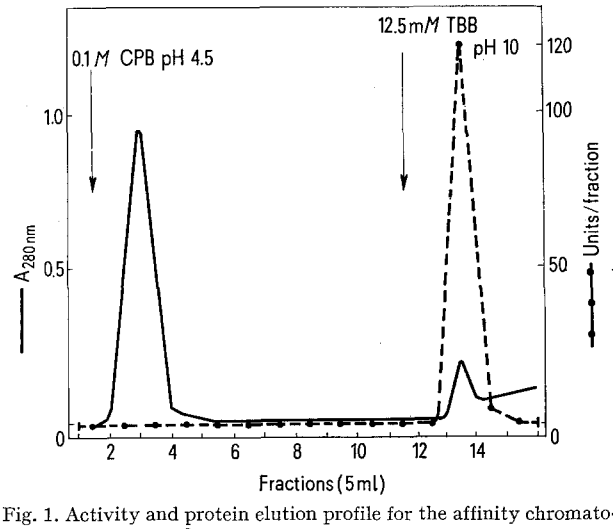

Fig. 1. Activity and protein elution profile for the affinity chromatography of N-acetyl-β-D-hexosaminidase.

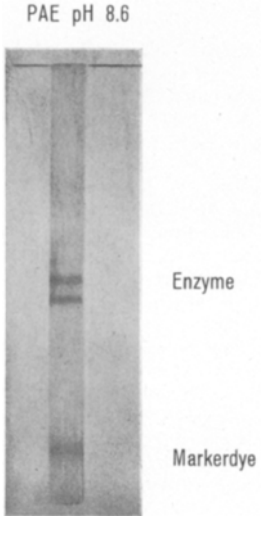

Fig. 2. Polyacrylamide gel of 10 µg N-acetyl-β-D-hexosaminidase run in the basic ORNSTEIN DAVIS[9] buffer system (7% gel).

Epididymes were obtained at castration or slaughter of adult boars, cut into segments and stored at −20 °C until used. The tissue was homogenized in 10 m*M* potassium phosphate buffer with an Ultra-Turrax homogenizer, centrifuged at 105,000 × *g* and the clear supernatant was used after dialysis for the experiments. Protein was determined by the method of LOWRY et al.[6] with crystalline bovine serum albumine as standard. The enzyme assay was a slight modification of the method described by LEVVY and CONCHIE[7]. One unit of enzyme activity is defined as that amount which is required to catalyze the formation of 1 µmol *p*-nitrophenol/min under the assay conditions. The specific activity of the enzyme was expressed as units per mg of protein.

Figure 1 shows the elution pattern of the enzyme from the affinity column. 0.7 ml of boar epididymis homogenate containing 5 mg of protein were dialyzed for 16 h at 4 °C against several liters of 0.1 *M* citrate phosphate buffer, pH 4.5. This material was applied to a 0.9 × 13 cm column containing the adsorbent. After washing the column with the citrate phosphate buffer elution was achieved with 12.5 m*M* borate buffer pH 10.0 (arrow) at a flow rate of approximately 20 ml/h. The pH of the eluted sample was immediately lowered to pH 4.5 with 10 volumes of 0.1 *M* citrate phosphate buffer. When a homogenate from boar epididymis was passed through the affinity column, N-acetyl-β-D-hexosaminidase was selectively retarded on the column.

Initially 4 other glycosidases are present in the epididymis homogenate: β-galactosidase, α-mannosidase, β-glucuronidase and α-fucosidase. These enzymes were not bound by the adsorbent to the column and were eluted in the first peak (Figure 1.) The separation of these enzymes by conventional purification procedures are tedious and cumbersome. This method of separation therefore offers great advantages. The homogenate showed a specific activity of 53 units N-acetyl-hexosaminidase/mg protein, which was increased about 35-fold in the fraction with the eluted enzyme, where the specific activity was about 1750 U/mg protein.

Purification of N-acetyl-β-D-hexosaminidase (NAH)

Sample	NAH (U/ml)	Protein (mg/ml)	Specific activity (U/mg)
Original	372.5	7.05	53.0
Fraction 2 + 3	0.012	0.17	0.07
Fraction 13	24.0	0.016	1750.0

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[5] P. CUATRECASAS and J. PARIKH, Biochemistry *11*, 2291 (1972).
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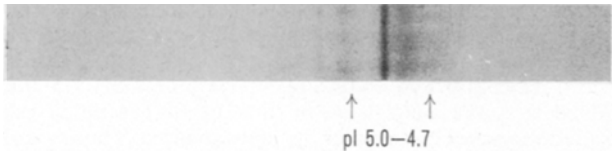

Fig. 3. Gel electrofocusing of N-acetyl-β-D-hexosaminidase according to AWDEH et al.[10]. The enzyme and carrier ampholytes (range pH 3–10) were set in the gel. Gels were removed and fixed in 5% trichloroacetic acid after focusing for 150 min at 50 mA (decreasing to 28 mA) at a voltage of 210 V (increasing to 1080 V). After the run, the pH gradient established was determined with a combined microsurface electrode.

During the purification procedure an appreciable amount of protein precipitated in the affinity gel. Despite this fact we had an overall recovery of more than 40% of the enzymatic activity.

The dialyzed fraction No. 13 was lyophilized and further characterized. On a Sephadex G 200 column, calibrated according to ANDREWS[8], the purified enzyme separated in 2 distinct peaks of MW 140,000 and 65,000 daltons. Most of the enzyme activity was associated with the peak of 140,000 daltons.

Polyacrylamide-gel electrophoresis[9] showed it still to be heterogenous and revealed 2 major bands in close vicinity (Figure 2.) After cutting the gel into 1 mm discs and eluting them with 0.1 *M* CPB pH 4.5, two fractions with enzyme activity were detectable, which corresponded with the protein bands in the gel. Isoelectrofocusing in thin-layer-polyacrylamide-gel[10] revealed microheterogeneity of the enzyme preparation, showing a pI region of pH 4.7–5.0 (Figure 3).

BULLOCK and WINCHESTER[11] and WINCHESTER[12] demonstrated several components of N-acetylhexosaminidase in ram epididymis by isoelectric focusing. They found in distinct anatomical sections of the ram epididymis different predominant N-acetylhexosaminidase components. In our case, using a *p*-aminophenyl-N-acetyl-β-D-thioglucosaminid as the inhibitor coupled to Sepharose 4 B, we gained a 35-fold purification of N-acetylhexosaminidase from boar epididymis.

With this method of affinity chromatography, we devized a technique to isolate minute amounts of N-acetylhexosaminidase to a fair degree of purity from the different segments of the boar epididymis and to study their possible significance on sperm maturation.

Zusammenfassung. Aus dem Nebenhoden vom Eber gewonnene N-Acetylhexosaminidase (EC 3.2.1.30) wurde mittels Affinitätschromatographie etwa 35fach gereinigt. An einen Träger gebundenes *p*-Aminophenyl-Thioglucosaminid diente als reversibles Adsorbens für das Enzym. Einige physicochemische Charakteristika wurden bestimmt: Molekulargewicht 140.000; pI-Bereich 4,7–5,0.

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Enhanced Calcium Accumulation Related to Increased Protein Phosphorylation in Cardiac Sarcoplasmic Reticulum Induced by Cyclic 3′, 5′-AMP or Isoproterenol

Catecholamines have been demonstrated to increase Ca-transport in cardiac sarcoplasmic reticulum (SR), as shown by EBASHI and ENDO[1], SHINEBOURNE et al.[2] and others. This effect involved the following sequence of processes: activation of adenylate cyclase, cyclic adenosine 3′, 5′-monophosphate (cAMP) synthesis and a cAMP and protein kinase (PK) EC.2.7.1.37 PK-mediated phosphorylation of SR protein[3–5]. The latter step is believed to be directly responsible for changes in the rate of calcium transport[6–8].

In the present study, evidence is given of the quantitative relationship between demonstration of the above sequence of processes in vitro, when applying cAMP to the isolated vesicles of SR and the effects of in vivo application of isoproterenol (ISO).

Material and methods. Male adult mongrel dogs were used, injected with a single dose of 7.5 mg/kg ISO in saline. Cardiac microsomes were prepared from the ventricles by the method of HARIGAYA and SCHWARTZ[9]. Electron microscopic examination of the membranes showed homogenous vesicles without evidence of mitochondria.

The activities of the marker enzymes glucose-6-phosphatase EC.3.1.3.9, 5′-nucleotidase EC.3.1.3.5 and cytochrome c oxidase EC.1.9.3.1 were determined according to the method of BERGMEYER[10], MUIR et al.[11] and SMITH and CAMERINO[12].

The activities of these enzymes in preparations of high purity showed no significant differences ($p \geqslant 0.05$) between the preparations isolated from the controls and from

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