MS, a demyelinating disease in which the impairment of blood-brain barrier is well documented[4,5]. At the present stage it cannot be argued that the serum factors responsible for the generation of DM ultimately cause myelin disintegration, however they may contribute to the process by rendering the sheath more susceptible to other humoral and cell-mediated demyelinating agents. The above in conjunction with augmented myelinolytic activity of sera from MS patients[17-19] is likely to contribute to the pathogenesis of this disease.

In conclusion, the present study demonstrates that the CNS myelin in vivo is vulnerable to some components present in normal serum. By as yet unknown mechanisms these factors induce membrane changes in the sheath leading to the generation of low density fragments (DM). The increased recovery of DM from the tissue homogenates seems to be an early and sensitive index of serum-induced myelin alterations.

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Trypsin in human milk

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Summary. Human milk trypsin was purified by adsorption chromatography on cellulose-bound 4-aminobenzamidine; its molecular weight was about 24,000 daltons. Its concentration determined by a radioimmunoassay varies between 2.9 and 5.6 µg/l.
Key words. Human milk; trypsin; affinity chromatography; HPLC; radioimmunoassay.

Human milk contains a larger amount of proteolytic enzymes than cow milk[1] but the identity of the proteases is not yet completely established. Only Borulf et al. reported the isolation of a trypsin-like enzyme from human colostrum by immunoadsorption chromatography[2]. In the present note we report the trypsin content of human milk determined by a radioimmunoassay and the purification of the enzyme from human colostrum and milk by adsorption chromatography on cellulose-bound 4-aminobenzamidine, followed by high pressure liquid chromatography on a TSK-SW-type column.

Materials and methods. Human milk samples from healthy mothers were obtained at the hospital and from individual donors. Milks were defatted by centrifugation and frozen until use. Radioimmunoassay was performed according to Temler and Felber[3] with trypsin-specific immunochemicals from Serono Diagnostics (Coinsins, Switzerland): the antibodies did not cross-react with chymotrypsin (EC 3.4.21.1), thrombin (EC 3.4.21.5), plasmin (EC 3.4.21.7) and elastase (EC 3.4.21.11). Affinity chromatography on cellulose-bound 4-aminobenzamidine was performed as indicated elsewhere[4]. 0.5 ml of moist adsorbent was mixed with 100 ml milk and gently stirred for 3 h at room temperature. The desorbed material was dialyzed against 10 mM HCl. Active trypsin was determined by the procedure elsewhere described[4]. To detect whether the enzyme was active in milk, 1 ml of 10 mM BAPA (benzoyl-L-arginine p-nitroanalide) reagent was added to 10 ml of a milk sample. After incubation for 12 h at 37°C, 200 µl of 24% trichloroacetic acid were added and the precipitate removed by centrifugation (20,000 g; 30 min). The supernatant was filtered through Millipore 0.22 µm filters (Millex-HC, Millipore, Bedford) and measured at 410 nm.

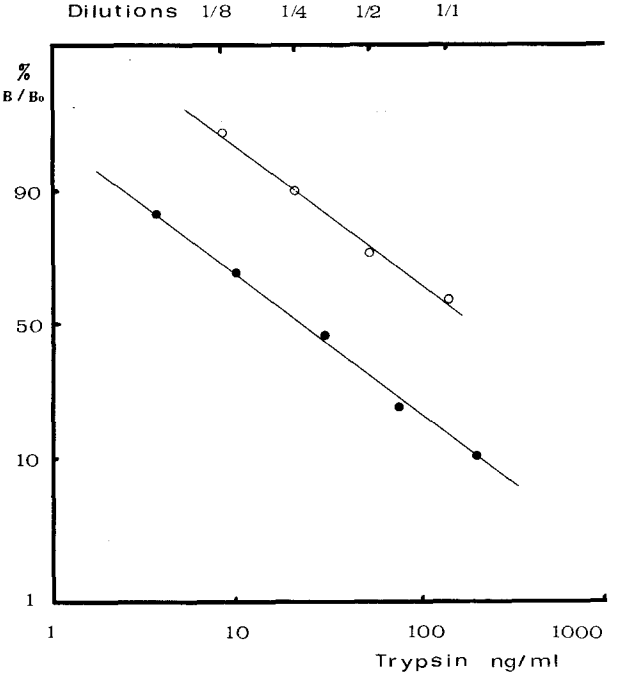

Figure 1. Logit plot of the reference calibration (curve) (●) and serial dilution of a pool of milks (○). B_0, radioactivity in absence of unlabeled antigen. B, radioactivity of the bound fraction.

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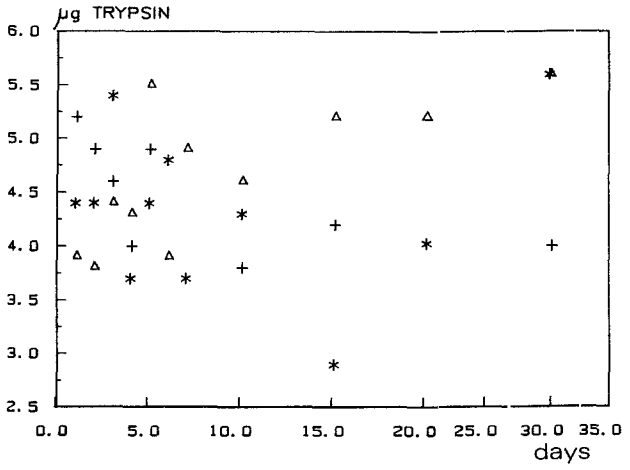

Figure 2. Human milk trypsin distribution during lactation determined by radioimmunoassay. Different symbols correspond to different milk samples.

HPLC was carried out on a Varian 5000 liquid chromatograph equipped with a TSK-2000-SW column, with 50 mM TRIS/HCl pH 7.0, 10 mM $CaCl_2$, 0.5 M NaCl, as eluent. The detector was fixed at 220 nm and the fraction collector was a Gilson 201 model. Cellulose-bound 4-aminobenzamidine and chemicals for buffer preparation were from Merck, (Darmstadt, Germany). Proteins were from SIGMA (St. Louis, USA) and porcine crystalline trypsin (EC 3.4.21.4) was a 4500 K NOVO preparation.

Results. Trypsin detection by radioimmunoassay (RIA): A RIA developped to detect trypsin in human blood plasma enabled us to determine the enzyme directly in defatted milk samples; in fact, response rate on a dose curve obtained with a pool of five milks in four successive dilutions was strictly parallel to the reference standard curve obtained with human trypsin (fig. 1). The trypsin content of three different milk samples was followed during the first month of lactation, more particularly during the first 10 days; it varied between 2.9 and 5.6 µg trypsin/1 milk. We have not so far found any correlation between enzyme concentration and lactation time (fig. 2).

Trypsin activity determined after direct addition of BAPA into the milk. For details see experimental section

Sample	Absorbance at 410 nm	Amount of enzyme*
Milk 1	0.144	25 ng/100 ml
Milk 2	0.021	n.d.

*Porcine trypsin was used as a standard; n.d., not detectable.

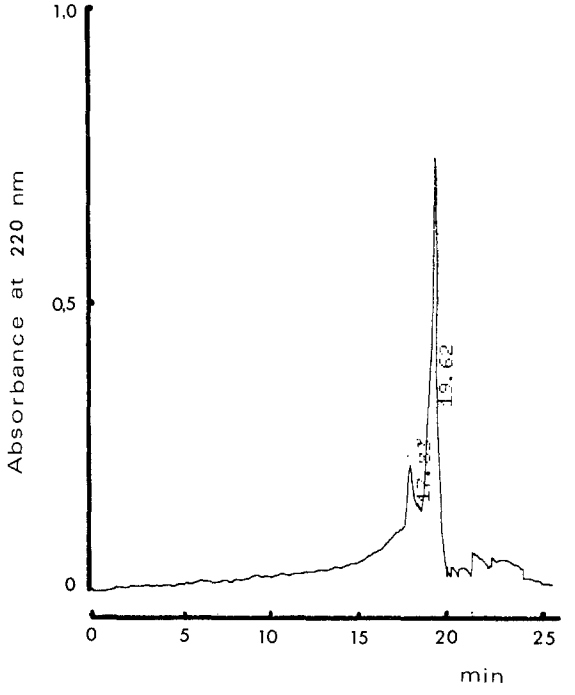

Figure 3. HPLC pattern of the material eluted from the 4-aminobenzamidine column. Absorbance (220 nm) vs retention time (min).

Characterization of free trypsin: Tryptic activity was determined as indicated in the experimental section. In most of the samples the synthetic substrate was not at all hydrolyzed because in only one quarter of the samples analyzed (30 milks) a significant difference between the blank and sample was detected spectrophotometrically. The table shows the difference between a milk which contains detectable active enzyme and another which does not.

Affinity chromatography and HPLC of human trypsin: By adsorption chromatography between 150 ng and 250 ng of active enzyme were obtained per 100 ml of skimmed milk: these figures were inferred from a standard curve established with porcine trypsin.

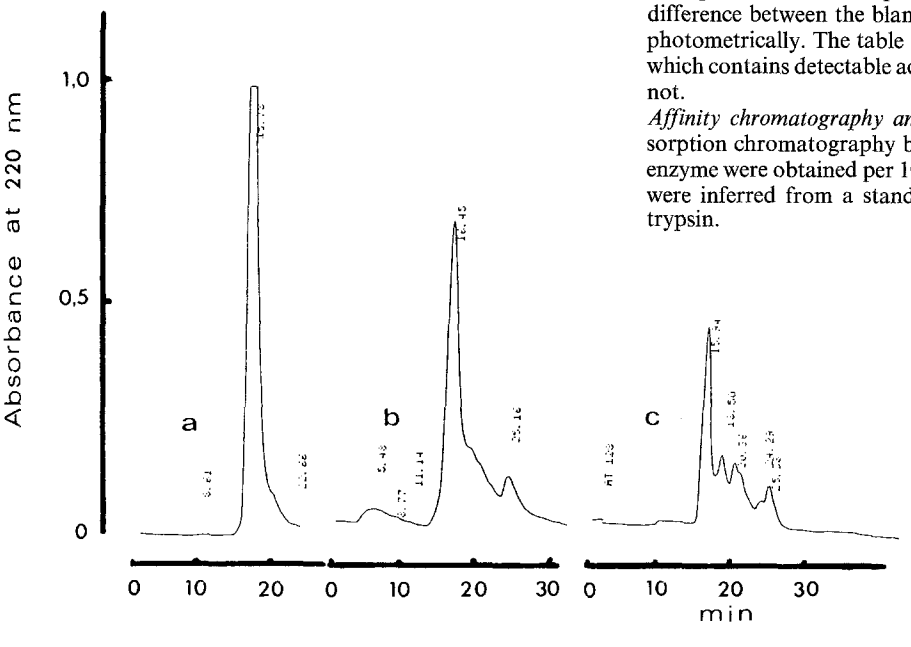

Figure 4. HPLC pattern of cow β-lactoglobulin digestion by human milk trypsin. Absorbance (220 nm) vs retention time (min) (a) 0 min, (b) 4 h at 40 °C, (c) 17 h at 40 °C. TRIS/HCl 50 mM buffer, pH 7.8, 10 mM $CaCl_2$. The E/S ratio was approximately 0.1/100.

By gel permeation HPLC it could be shown that the material obtained by affinity chromatography was mainly (80% of the total area) a protein with a molecular weight of about 24 kDa as it eluted at the same retention time as bovine trypsinogen employed as a marker (fig. 3). Only the main HLPC fraction which contained the enzyme was automatically collected and, after dialysis and lyophylization, bovine β-lactoglobulin was added: the latter was digested to an important extent by the purified enzyme (fig. 4).

Discussion. It has been suggested that digestive assistance to the newborn constitutes one of the roles of human milk proteases[1]. We found in this study that trypsin was fully active in hydrolizing BAPA and cow β-lactoglobulin once the enzyme was extracted from the milk by affinity chromatography. However, when BAPA was directly allowed to react with milk, the chromogenic substrate was hydrolyzed by only a few samples. This observation is in accordance with the findings of Lindberg et al.[5], who found no protease-inhibiting activity in one-third of the milks they analyzed.

It is noteworthy that trypsin was detected in all the milks analyzed in our laboratory by the radioimmunoassay: the antibodies utilized recognized the enzyme even when it was blocked by specific inhibitors (such as α_1-antitrypsin in blood serum). Since trypsin concentration in normal blood serum varies between 26–53 µg/l[3], it can be assumed that the pancreatic enzyme passes from the blood stream into the milk.

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Eyestalks control of diurnal rhythm of acetylcholinesterase activity in the crab *Oziotelphusa senex senex*[1]

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Summary. The crab *(Oziotelphusa)* displays a diurnal rhythm of acetylcholinesterase activity, with maximal activity around midnight, alternating with minimal activity at noon. Bilateral eyestalk ablation eliminates the diurnal rhythm of acetylcholinesterase activity.

Key words. Crab; *Oziotelphusa;* diurnal rhythm; acetylcholinesterase; eyestalk; thoracic ganglion.

Rhythmic variations in acetylcholinesterase activity, with peak periods of activity during dark periods, have been found in nocturnal animals like scorpions[2], cockroaches[3], snails[4] and slugs[5]. The crab *(Oziotelphusa)* also displays a diurnal rhythm of acetylcholinesterase (AChE) activity[6]. The neurodepressing hormone (NDH) of eyestalks of the crab *Oziotelphusa* has been isolated and characterized as a peptide; it is involved in the control of acetylcholinesterase activity. We therefore undertook to investigate the possibility that the role of eyestalks (perhaps NDH) in controlling the rhythm of AChE activity is attributable to its effects of diurnal organization.

Materials and methods. Adult healthy male specimens (30–32 g) were collected from local paddy fields. They were kept singly in 1000-ml glass aquaria and acclimated for 20 days to laboratory conditions (temp. 28 ± 2°C) under a 12:12 (06.00–18.00; 18.00–06.00 h) light:dark regimen. The crabs were fed twice weekly with frog muscle and the medium was changed daily. In the present study only intermolt[8] (stage C_4) crabs were selected and grouped into four groups of 150 each as follows: Group 1: normal. Group 2: control, sham-operated. Group 3: control, crabs kept in continuous darkness (DD). Group 4: bilateral eyestalk ablation (ESX). The eyestalks of the crabs, after anesthetizing them by cooling in ice for 3 min, were excised by cutting off the organs at the base without prior ligation and the stubs were cauterized with a hot needle.

The thoracic ganglionic masses were isolated from all the crabs at different times of the day in cold crustacean Ringer's solution[9] and kept for 5 min, for recovery. In the present study, 6 time points; 08.00, 12.00, 16.00, 20.00, 24.00 and 04.00 h were selected for experimentation to cover the 24-h period of the day and at each time five animals were analyzed. AChE activity was assayed[10] and expressed as µmoles of ACh hydrolyzed/mg protein/h.

Results. The present study shows a diurnal rhythm of AChE activity in the thoracic ganglionic mass of *Oziotelphusa* (fig.). The AChE activity was at a minimum at noon (12.00 h). The activity showed a gradual increase as night approached, reaching a maximum at midnight (24.00 h) (fig., A). From this point onwards the activity showed a gradual decline, reaching a minimum at noon (12.00 h). The average change from maximal to minimal was −43.4%, and that from minimal to maximal was +81.5%. The differences between crests and troughs are statistically significant ($p < 0.001$). Bilateral eyestalk ablation eliminated rhythmic changes in AChE activity in all crabs examined (fig., D). In all the cases the loss of rhythmicity was evident within 4 h after operation. There was no evidence for rhythmic changes in AChE activity for at least 5 days after operation. Sham operation briefly disrupted the AChE rhythm for 4–8 h, then the rhythm of activity continued with unaltered amplitude and phase (fig., B). Crabs kept in continuous darkness (DD). which served as controls, also exhibited a rhythm of AChE activity with a maximum at midnight and a minimum at noon (fig., C). There was no mortality in normal, control or eyestalk-ablated crabs throughout the experiment.

Discussion. Under 12:12 L:D conditions, the intact crabs displayed a diurnal rhythm of AChE activity. Eyestalk ablation eliminated this rhythm but sham opration and keeping the crabs in continuous darkness had no effect. The elimination of rhythmicity can, therefore, be interpreted as the result of eyestalk ablation and not of surgical stress or associated experimental manipulations.

Eyestalk extirpation is a classical operation of crustacean endocrinology; it removes the X-organ sinus gland complex; the sinus gland is a neurohemal organ containing neuronal endings of the neuroendocrine system. The X-organ is the source of an array of hormones[11], which includes the recently demonstrated neurodepressing hormone (NDH). Removal of eyestalks results in elevation of neuronal activity, as demonstrated by the elevation of AChE[7] and spontaneous electrical activity[12]. Evidence for a circadian cycle in the neurosecretory activity of the sinus gland of *Astacus leptodactylus* has been demonstrated[13]. Ultrastructural