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.

1 Shahani, K.M., Kwan, A.J., and Friend, B.A., Am. J. clin. Nutr. *33* (1980) 1861.
2 Borulf, S., Hegardt, P., and Lindberg, T., Pediat. Res. *16* (1982) 12, 1041.
3 Temler, R.S., and Felber, J.P., Biochim. Biophys. Acta *445* (1976) 720.
4 Monti, J.C., and Jost, R., Biotech. Bioeng. *20* (1978) 1173.
5 Lindberg, T., Ohlsson, K., and Weström, B., Pediat. Res. *16* (1982) 479.

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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.

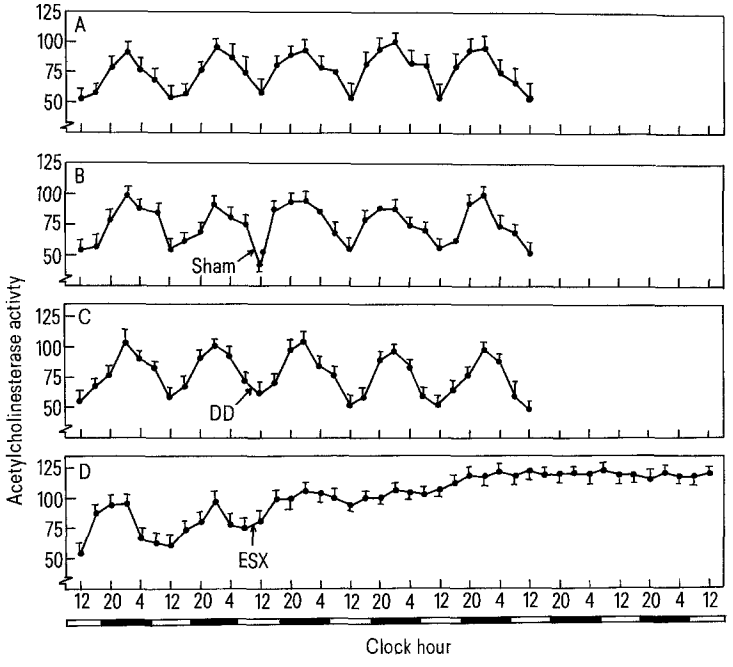
Materials and methods. Adult healthy male specimens (30–32 g) were collected from local paddy fields. They were kept singly in 1000-ml glass aquaria and acclimated for 20 days to laboratory conditions (temp. 28 ± 2°C) under a 12:12 (06.00–18.00; 18.00–06.00 h) light:dark regimen. The crabs were fed twice 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



A Changes in AChE activity in the thoracic ganglionic mass of intact crab over a period of 5 days. *B* Changes in AChE activity in the thoracic ganglionic mass of crab before and after sham operation. *C* Changes in AChE activity in the thoracic ganglionic mass of crab before and after keeping it in complete darkness (DD). *D* Changes in AChE activity in the thoracic ganglionic mass of crab before and after bilateral eyestalk ablation (ESX). AChE activity was measured at 4-h time intervals and are expressed as μmoles of ACh hydrolyzed/mg protein/h. Each point is the mean of five individual observations. Vertical bars represent standard deviation. Operations were performed at the indicated times.

investigations of the sinus gland show that the number of vesicles is lower in the axon terminals between 19.00 and 20.00 h. Furthermore sinus gland homogenates, quantitatively analyzed by polyacrylamide-gradient-gel-electrophoresis in microgels, indicate a decline in hormone content at the transition from light to darkness with a minimum at 22.00 h. It is reasonable, therefore, to suggest that the abolition of rhythmic change of AChE activity by eyestalk ablation in the present study could have resulted from a disruption of an endogenous diurnal variation in hormonal and neuronal activity that affects AChE activity.

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2 Venkatachari, S.A.T., and Muralikrishna Das, P.M., Life. Sci. *7* (1968) 617.
3 Vijayalakshmi, S., Murali Mohan, P., and Sasira Babu, K., J. Insect Physiol. *23* (1977) 195.
4 Reddy, G.R., Pavankumar, T., Murali Mohan, P., and Sasira Babu, K., J. comp. Physiol. *125* (1978) 59.
5 Pavankumar, T., and Sasira Babu, K., Experientia *34* (1978) 61.
6 Surendra Reddy, K.V., M. Phil. thesis., S.V. University, Tirupati, 1978.
7 Sreenivasula Reddy, P., and Ramamurthi, R., Geobios, in press (1985).
8 Drach, P., Ann. Inst. Oceanogr. Paris *19* (1939) 103.
9 Van Harreveld, A., Proc. Soc. exp. Biol. Med. *34* (1936) 428.
10 Glick, D., Methods in Biochemical Analysis, vol. 5, p. 1. Interscience Publishers Inc., New York 1957.
11 Kleinholz, L.H., Am. Zool. *6* (1966) 161.
12 Arechiga, H., Huberman, A., and Martinez Palomo, A., Brain. Res. *128* (1977) 93.
13 Strolenberg, G., Van Herp, F., Van Wormhoudt, A., and Bellon-Humbert, C., in: Comparative Endocrinology, p. 173. Eds P.J. Gaillard and H.H. Boer. Elsevier/North-Holland Biomedical Press, Amsterdam 1978.

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Interaction of *Rhizobium loti* strain and host on the biosynthesis of unusual amino acids in leguminous plants

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Summary. The effect of different *Rhizobium loti* strains on the biosynthesis of 2,3-diamino-butanoic acid and 2,4-diamino-3-methyl-butanoic acid in root nodules of *Lotus tenuis, Anthyllis vulneraria* and *Lupinus densiflorus* has been investigated. Results suggest that biosynthesis is *Rhizobium* strain dependent, that the bacteroid is the site of synthesis of the compounds and that their biosynthesis is confined to the symbiosis.
Key words. 2,3-Diamino-butanoic acid; 2,4-diamino-3-methyl-butanoic acid; biosynthesis; *Rhizobium loti;* leguminous plants.

It has been known for some time that leguminous root nodules contain, in addition to the common amino acids, a number of ninhydrin positive compounds of unusual Rf values[1,2] as determined by paper chromatography. The synthesis of these unusual amino acids in *Lotus*[1] and a range of other leguminous species[2] was shown to be *Rhizobium* strain dependent. Recently, two of the unusual amino acids found in *L. tenuis* nodules have been identified as 2,4-diamino-3-methyl-butanoic acid[3] and 2,3-diamino-butanoic acid[4]. The former compound was found in large amounts in *L. tenuis* nodules containing *R. loti* strains NZP2227 or NZP2238 and the latter was abundant in *L. tenuis* inoculated with NZP2213. In order to confirm that the biosynthesis of these two unusual amino acids is *Rhizobium* strain dependent, we have examined the amino acid content of nodules from three different leguminous species inoculated with the different *R. loti* strains.

Materials and methods. Rhizobium strains and growth conditions.