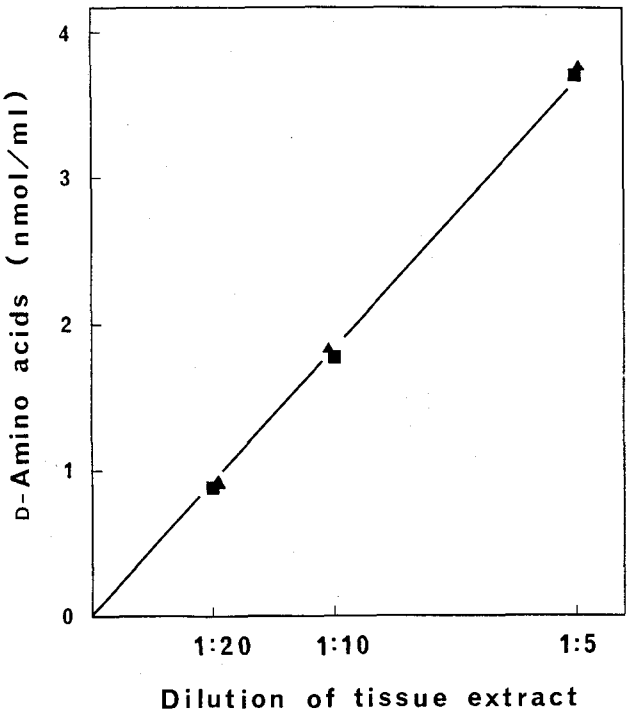

Figure 2. Dilution of tissue extracts vs. their inherent free D-amino acid concentration. Free D-amino acids were measured after dilution of kidney (▲) or liver (■) extract with PBS 1:5, 1:10 or 1:20, and the D-amino acid concentration of the diluted extract was obtained.

Discussion. The present assay method detects only D-isomers of free neutral amino acids such as Ala, Met, Pro, Tyr, Ile, Leu, Phe, Ser, Val and Trp, and also Gly and L-Pro weakly, because of the specificity of DAO[8]. The rate of color formation due to Gly was 1/200 of that due to D-Ala, and that due to L-Pro was far less than that for Gly in our assay. Gly and L-Pro are not likely to have been measured as DAA, because there was no difference in the free Gly and Pro levels (Hitachi Amino Acid Analyser L8500) of the kidney between the DAO-lacking mutant and the control mice, whereas the free DAA level of the mutant was measured to be 6-fold higher than that of the control (data not shown).

The amino acids observed in the present study were probably present as free amino acids, because no enzyme has been found in mammals that can hydrolyze peptides containing DAA residues. In addition, only the D-form of amino acids present in the tissues could have been measured, since no racemase that might produce DAA from their L-enantiomers is known in mammals. It is not clear whether the DAA are of endogenous origin, or come from exogenous sources like gut bacteria.

The existence of free DAA has been demonstrated in animal tissues. This suggests that the physiological role of DAO is, in part, to catalyze the oxidative deamination of free DAA. The physiological role of the enzyme remains obscure, in spite of its wide distribution in various organs of many animals.

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Prothymosin alpha is not a nuclear polypeptide

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Summary. Using a radioimmunoassay for the NH_2-terminus of prothymosin alpha, the crossreactive material was measured in subcellular fractions of calf thymus and liver. No significant amount of crossreactive material was found in the nucleus. This provides experimental evidence against a recent hypothesis, based on structural evidence, that prothymosin alpha is a nuclear polypeptide.
Key words. Prothymosin alpha; thymosin alpha 1.

Prothymosin alpha is a polypeptide of approximately 110 residues which has been isolated and sequenced from mammalian tissues[1-5]. It has a wide tissue[6-8] and phylogenetic distribution[9,10]. It has been related to cell

growth[4] and cell-mediated immunity[11-13]. Although the biological role of prothymosin alpha is far from being known yet, its wide distribution suggests that it has an important one.

Recently a hypothesis was put forward, based mainly on the published structural properties of prothymosin alpha, according to which this polypeptide is located in the nucleus[14]. We provide here experimental evidence suggesting that prothymosin alpha is not a nuclear polypeptide.

Materials and methods. For the isolation of the nuclei, the citric acid method[15] was followed. Liver and thymus were collected from an approximately 18-month-old calf immediately after slaughter. Tissues were carried to the laboratory on ice and cut into small pieces. Quantities of 10 g of thymus and liver were homogenized with 10 vol. of 2.5% citric acid solution in a Sorval Omni Mix blender for 10 and 5 s respectively. Each homogenate was centrifuged in a Sorvall RC 5C centrifuge at 1000 × g for 10 min. The cytoplasmic supernatant designated (S) was saved for subsequent heat treatment and the pellet containing the nuclei, possibly contaminated by unbroken cells, was dispersed in 20 ml of solution A (0.25 M sucrose in 1.5% citric acid). The suspension was centrifuged as above. The pellet enriched in nuclei was dispersed in 20 ml of solution A and the sample was superimposed on 30 ml of 0.88 M sucrose in 1.5% citric acid. After centrifugation as above the pellet containing the nuclei, as observed by light microscopy, was dispersed in solution A to a final volume of 10 ml. The nuclear preparation was designated (N). The above procedure was carried out at 4 °C.

Each thymic and liver (S) and (N) preparation was added to an equal volume of boiling water. Boiling continued for 5 min more after the addition of the sample. This heat treatment was carried out for the elimination of continuing proteolysis, which is known to reduce prothymosin alpha to shorter fragments[16, 17]. All samples were sonicated using an MSE sonicator. The duration of sonication was 1 min for both (S) and 2 and 3 min for liver and thymus (N) samples. Lysis of nuclei was virtually complete. The samples were centrifuged in a Sorval RC 2B centrifuge at 12,000 × g for 10 min and the supernatants were desalted and concentrated by Sep-Pak C18 cartridges (Waters Ass., USA). Each sample was divided into 10 equal aliquots and each aliquot was forced, using a syringe, through 3 cartridges serially connected by rubber tubing.

The bound peptides in each set of 3 cartridges were eluted with 10 ml of 25% propanol in 1 M HCOOH/0.2 M pyridine, pH 2.8. Aliquots of 0.2–2 ml of the eluted material were lyophilized in a Savant Speed-Vac concentrator and crossreactive material measured by a radioimmunoassay specific for the NH_2-terminus of prothymosin alpha[10]. Polyclonal antibodies crossreacting with prothymosin alpha were generated against thymosin alpha 1 (fragment 1-28 of the NH_2-terminus of prothymosin alpha), and prothymosin alpha (1-28) (^{125}I) was used as the tracer in the radioimmunoassay. The standard curve was constructed with thymosin alpha 1. Controls of whole tissue extracts designated (C) were prepared by blender homogenization of 3 g of tissue with 10 vol. of buffer A in three 45-s bursts at full speed. The homogenate was kept on ice for the whole duration of the preparation of (S) and (N) fractions (approximately 1 h). This was followed by boiling in an equal volume of water for 5 min, and by 2 and 5 min of sonication for liver and thymus tissues respectively. Subsequent concentration and desalting of (C) was carried out as described above for (S) and (N) samples.

Results and discussion. Calf tissues were used as they are easily available in large quantities and calf prothymosin alpha has recently been isolated[8, 17] and sequenced[5], showing a strong homology to the known sequences of the human[2-4] and rat[1] polypeptides. The levels of prothymosin alpha cross-reactive material in the nucleus were found to represent less than 1% of the cytoplasmic levels in both thymus and liver (table). These results suggest that prothymosin alpha does not accumulate in the nucleus, as claimed in a recent hypothesis[14].

The cytoplasmic levels in thymus and liver accounted for 32 and 47% of the maximum extractable whole tissue crossreactive material (table). The discrepancy between these levels and the total crossreactive material in both tissues possibly reflects to some extent the milder cell breakage procedure employed for the isolation of nuclei. The levels of crossreactive material in thymus extracts were found to be 4.5 times higher than in liver extracts (table) in agreement with results obtained from rat tissues (6 times higher)[6], but were lower (7 μg/g) than those previously reported (12.3 μg/g)[10] when proteolysis was immediately eliminated upon tissue excision.

Our results are in agreement with the detection, by monoclonal antibody against thymosin alpha 1 and immunocytochemical techniques, of crossreactive material in the cytoplasm and/or cytoplasmic vacuoles of thymic reticuloepithelial cells[19, 20]. It would be interesting to know whether other thymosins (parathymosin alpha[11], and thymosins beta 4, beta 9, beta 10 and beta 11[21]) share the same intracellular distribution with prothymosin alpha.

Levels of prothymosin alpha crossreactive material in cellular fractions of calf tissues

Tissue	Fraction[a]	Levels[b]	Percentage[c]
Thymus	C	6994 ± 1450	100
	S	2250 ± 373	32.2
	N	10.1 ± 1.5	0.1
Liver	C	1558 ± 381	100
	S	726 ± 60	46.6
	N	3.3 ± 1.3	0.2

[a] C, control, whole tissue extracts; S, extranuclear material; N, nuclear material. [b] Expressed in ng of thymosin alpha 1 equiv./g of wet tissue. Thymosin alpha 1 is the NH_2-terminal segment 1-28 of prothymosin alpha. Results are from at least triplicate analyses. [c] Percentage of crossreacted material in (C).

The cytoplasmic location of prothymosin alpha is in agreement with the known release of prothymosin alpha in blood plasma [8] and thymosin alpha 1 crossreactive material in serum [22] by an as yet unknown mechanism. It is speculated that thymosin beta 4 is also a cytoplasmic polypeptide as it has been found in human plasma at higher than prothymosin alpha levels [23].

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β-Methyl carboline, a benzodiazepine inverse agonist, attenuates the effect of triazolam on the circadian rhythm of locomotor activity

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Summary. The benzodiazepine triazolam, the benzodiazepine inverse agonist, β-methyl carboline (β-CCM) or both, were administered to adult male hamsters under conditions of constant light. When given alone, triazolam induced phase advances in the circadian activity rhythm of about 90 min, while β-CCM when given alone, had no effect on phase of the activity rhythm. However, when triazolam and β-CCM were given at the same time, the magnitude of the phase advances induced by triazolam were attenuated to about 30 min. These data, in conjunction with previous results, provide pharmacological evidence for a GABAergic system involved in the regulation of a central circadian pacemaker.
Key words. Benzodiazepine; circadian rhythm; gamma-aminobutyric acid; inverse agonist; suprachiasmatic nucleus; triazolam.

In the absence of environmental time cues, a single intraperitoneal injection of the relatively short-acting benzodiazepine, triazolam, can induce either phase advances or phase delays in the circadian rhythm of the onset of locomotor activity in the golden hamster. Whether an advance or a delay occurs in response to triazolam depends upon the phase of the animal's activity cycle at which the drug is administered: phase advances of the activity rhythm can be induced if an injection of triazolam is administered within 6 h before the onset of activity, while phase delays can be induced if triazolam is given 6–9 h after the onset of activity [1]. The phase-shifting effects of triazolam on the hamster activity rhythm are also dose-dependent [2] and can be blocked by Ro 15–1788, a benzodiazepine antagonist [3].

Triazolam is a member of the family of triazolobenzodiazepines that act by potentiating the effects of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) [4, 5]. A second triazolobenzodiazepine, midazolam, also induces phase advances and phase delays in