classified as metallo-proteases because of their sensitivity to o-phenanthroline and EDTA. Furthermore, dialysis of the carboxypeptidase at pH 5.5 first in the presence and then in the absence of EDTA completely abolished enzyme activity, which could be restored by micromolar amounts of Zn^{2+} and Co^{2+} but not of other divalent cations (data not shown).

As mentioned above, cell extract fractionation could resolve two aminopeptidase activities, I and II, the former being mainly active against Leu-pNA, the latter against both Leu-pNA and Arg-pNA. We tentatively propose, however, that these two activities represent different forms of the same enzyme. This is substantiated by the fact that they displayed practically identical inhibition patterns (table 1), stability curves (fig. 2), pH optima and activation energies (data not shown): furthermore, Hanner et al.[6] could isolate only one form of aminopeptidase from *Sulfolobus solfataricus*, and its properties compare well with those of both forms described in this paper.

Determinations of protease levels in crude extracts evidenced substantially higher specific activities for the exopeptidases as compared to those of the endopeptidases: this suggests that the strategy of intracellular protein degradation of this microorganism might involve aspecific roles for the former enzymes and more specific ones for the latter. This is also supported by the fact that the exopeptidases proved to be active against several synthetic substrates, whereas the endopeptidases could only attack a limited number of them (data not shown).

In conclusion, the data presented here show that *Sulfolobus solfataricus* represents a rich source of proteolytic enzymes, most of which are endowed with a remarkable thermostability. Purification of some of them is currently being carried on in our laboratory, and their possible biotechnological applications are under investigation.

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Copper metabolism in the LEC rat: Involvement of induction of metallothionein and disposition of zinc and iron

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Abstract. The Cu concentration was about 40 times higher in the liver of LEC (Long-Evans with a cinnamon-like coat color) rats aged 77 days (227.5 ± 21.6 μg/g liver) than in Fischer rats (5.2 ± 0.1 μg/g liver). However, in the kidney and brain of the LEC rats, Cu concentrations were lower than in these organs of the Fischer rats. Cu concentration in the hepatic metallothionein fraction was about 130 times higher in the LEC rats than in the Fischer rats. The LEC rats showed markedly low concentrations of Cu in the serum and bile. It seems likely that excretion of Cu from the liver into the bile and blood (as ceruloplasmin) is inherently lacking in the LEC rat.
Key words. LEC rat; copper; metallothionein; ceruloplasmin; zinc; iron.

The LEC (Long-Evans with a cinnamon-like coat color) rat is an inbred strain which shows fulminant liver damage at three to four months after birth[1]. This hepatitis, characterized by sub-massive necrosis of hepatocytes with few inflammatory cell responses, occurs in all rats of this strain[2]. Furthermore, in rats which survive for a long time, there is a high incidence of hepatocellular carcinomas[3]. Although several hypotheses regarding the fulminant hepatitis and spontaneous carcinomas in the LEC rat have been proposed[4], the pathogenic mecha-

nism responsible for the development of this hepatitis has not yet been fully elucidated. Recently, an abnormal deposition of Cu in the liver of the LEC rats was reported [5]. In order to understand the onset of liver injury, we focused on the distribution of Cu, Zn and Fe in the liver, kidney, spleen, brain and intestine, and, in addition, on the induction of hepatic metallothionein.

Materials and methods

LEC female rats were bred in our animal quarters. They weighed 130 ± 3 g when used at 77 days after birth. Fischer strain female rats of the same age, weighing 168 ± 2 g, were used for comparison. After weaning, the rats were maintained on CMF-food (Oriental Yeast Co., Ltd. Tokyo, Japan) containing 14.5 µg/g Cu, and were killed by heart puncture. The liver, kidney, small intestine (20 cm from the pylorus), spleen and brain were removed and immediately washed in chilled 0.9% NaCl solution. About 0.5 g of liver, left kidney, intestine (2 cm of the upper part), brain and spleen were digested with a mixture of perchloric and nitric acids (1:5) and the digest diluted with deionized water. The metal contents were measured using a Hitachi 208 atomic absorption spectrophotometer (AAS) with an air/acetylene flame or a Hitachi 180-80 AAS with a graphite furnace. Serum and bile were assayed directly in the AAS after dilution with deionized water.

About 1 g of liver, or of mucosa from the intestine was homogenized, using a teflon-glass homogenizer, with 5 volumes of 0.25 M sucrose solution deaerated with N_2 gas. The homogenate was centrifuged at 100,000 × g for 60 min. The supernatant was used for isolation of metallothionein (MT) or for determining the distribution of Cu and Zn. Hepatic MT was determined by a modification of the method of Bartsch et al. [6]. Since the MT had been shown by chromatography on Sephadex to be a Cu-rich one, it could be assayed by measuring the Cu and Zn remaining in the cytosol after heat treatment (90 °C for 3 min) and the addition of acetonitrile. MT fractions obtained from a Sephadex G-75 column (1.5 × 60 cm) were lyophilized, applied to a DEAE-Sephadex A-25 column (1.5 × 60 cm), and eluted by a stepwise method with 0.1 M to 0.2 M Tris/HCl buffer (pH 8.5).

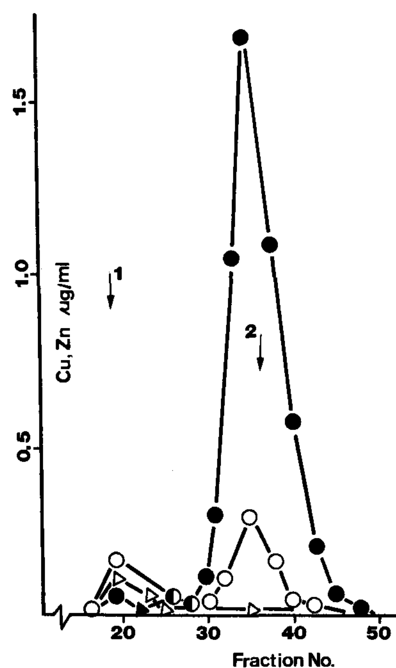
Biochemical evaluation of liver function was carried out by measuring serum enzyme activities of aspartate aminotransferase (GOT), alanine aminotransferase (GPT), lactate dehydrogenase (LDH) and alkaline phosphatase (ALP). Urea nitrogen (BUN) and creatinine (CRE) in serum were measured for evaluation of renal function. These parameters were measured with an automatic analyzer using commercial kits (Wako Pure Chemical Industries, Ltd. Osaka, Japan). Ceruloplasmin (Cp) activity was measured by the method of Schosinsky et al. [7]. Protein was determined by the method of Lowry et al. [8]. All chemicals used were of the highest purity commercially available. Results were analyzed by Student's t-test, taking $p < 0.01$ to be the level of significance.

Results

In the LEC rats, biochemical tests of liver (GOT, GPT and LDH) and kidney function (BUN and CRE) did not show any marked evidence of injury, although slight increases were found in GOT (68 ± 14 and 78 ± 3 Karmen Units in the Fischer and LEC rats, respectively) and GPT (29 ± 1 and 33 ± 1 Karmen Units in the Fischer and LEC rats, respectively). This showed that they had not yet developed hepatitis at 77 days after birth.

As shown in table 1, hepatic Cu deposits were more than 43 times higher in the LEC rats than in the Fischer rats. This was accompanied by 2.3- and 1.4-fold higher Zn and Fe concentrations, respectively. However, in the kidney and serum of the LEC rats, the Cu concentration was significantly lower (54% and 6.6%, respectively). The concentration of Fe in the spleen of LEC rats was also significantly lower (41%).

Distribution of Cu and Zn in the liver cytosol fraction was determined by column chromatography using Sephadex G-75. Typical distribution profiles of Cu and Zn are shown in the figure. Ninety-six percent of cytosolic Cu and 66% of the Zn (table 2) was found in the MT region (fig.). The Cu in this MT region was completely recovered even after heating at 90 °C for 5 min (data not shown). Further, the Zn in this peak could be displaced with Cd in vitro (data not shown). The molecular weight of this peak was estimated to be about 10 kDa, from its behavior on the Sephadex column. Therefore, the substance was considered to be a MT which is rich in Cu



Distribution of Cu and Zn in the liver cytosol fraction of the Fischer and LEC rats. Hepatic cytosols from the LEC and Fischer rats with the same protein content were applied to a Sephadex G-75 column (1.5 × 60 cm). ●—● and ○—○: Cu and Zn in the LEC rat, respectively. ▶—▶ and ▷—▷: Cu and Zn in the Fischer rat, respectively. Arrows 1 and 2: elution points of dextran blue and cytochrome c, respectively.

Table 1. Concentrations of Cu, Zn and Fe in organs and serum. Data: mean ± SD (μg/g wet wt or μg/ml[a]) of five rats. * $p < 0.01$.

		Liver	Kidney	Spleen	Intestine	Brain	Serum[a]
Fischer	Cu	5.2 ± 0.1	15.3 ± 3.5	1.4 ± 0.1	2.1 ± 0.1	2.7 ± 0.1	1.5 ± 0.0
	Zn	30.3 ± 0.8	24.9 ± 1.4	22.4 ± 1.0	25.3 ± 1.0	13.4 ± 0.4	1.1 ± 0.0
	Fe	155.9 ± 11.2	74.0 ± 6.2	753.4 ± 47.5	38.2 ± 4.0	20.6 ± 1.5	3.7 ± 0.6
LEC	Cu	227.5 ± 21.6*	8.3 ± 0.4*	1.2 ± 0.1	2.1 ± 0.2	2.4 ± 0.1	0.1 ± 0.0*
	Zn	68.8 ± 4.9*	22.0 ± 1.1	22.2 ± 1.3	24.1 ± 1.2	13.3 ± 0.3	1.4 ± 0.0*
	Fe	214.0 ± 10.2*	69.7 ± 4.5	307.0 ± 17.7*	38.9 ± 4.3	19.3 ± 0.7	3.7 ± 0.5

Table 2. Distribution of Cu in the liver and concentration of Cu and Zn in MT fraction. Data: mean ± SD of five rats. a: Cu or Zn μg/g protein. b: Cu was not detected in the MT region by AAS with flame. * $p < 0.01$.

	Cu		Cu, Zn-MT		Ratio (mol)
	Cytosol (%)	MT (%)	Cu[a]	Zn[a]	Cu/Zn
Fischer	64.8 ± 2.7	b	14.6 ± 1.6	52.8 ± 5.5	2/7.1 ± 0.8
LEC	84.6 ± 0.4*	95.5 ± 2.1	1844.9 ± 107.6*	316.0 ± 13.2*	5.9 ± 0.1/1

(table 2). The concentration of Cu in the MT fraction was about 130 times higher in the LEC rat than in the Fischer rat (table 2). The pooled MT-fractions obtained from the Sephadex G-75 were separated into three components by DEAE-Sephadex A-25.
Activities of two metalloenzymes were measured in the serum. There was no difference between the LEC and Fischer rats in the activity of alkaline phosphatase (ALP) (data not shown). However, the LEC rats showed a very low activity of serum Cp (3.1 ± 2.7 U/l) compared with the Fischer rats (172.3 ± 20.3 U/l).

Discussion

Wilson's disease is partly characterized by abnormal deposition of Cu in the liver, kidney, cornea and brain[9]. The Cp activity and Cu concentration are markedly lower in the serum of these patients as compared to normal individuals[10]. The distribution of Cu in the kidney and brain of the LEC rats (table 1) suggests that the Cu metabolism is different from that of Wilson's disease, although hepatic Cu showed a markedly high concentration (table 1), and Cp activity (as oxidase activity) was very low.
The hepatic Cu concentration was high not only in the cytosol fraction, but also in the 100,000 × g pellet. The proportion of Cu in the cytosol seemed to be a little higher than that in Cu-loaded animals[11]. The greater part of the Cu in the cytosol fraction was bound to MT. The MT in these rats may be similar to the MT obtained from Wilson's diesease patients[12] or from Cu-loaded rats[13].
The main route for excretion of Cu from the liver is in the bile as a low-molecular-weight compound[14]. We measured the Cu concentration in the bile of three LEC rats aged 100 days, in addition to those used in this series. The concentration was 0.12 ± 0.01 μg/ml bile, which was low when compared to the 0.59 ± 0.06 μg/ml bile of Wistar strain rats. In the LEC rat, the excretion of Cu into bile may be defective. In addition, the low concentration of serum Cu (table 1) may mean that Cu cannot be distributed to other organs. The synthesis of Cp may possibly be inherently defective in the LEC rat. We reported previously that hepatic MT-bound Cu was available to convert apo-Cp to Cp[15].
Generally, when an animal is over-exposed to Cu, to prevent Cu toxicosis, excretion of Cu into the bile increases and MT induction is enhanced[16]. Since the two routes mentioned above were nearly closed in the LEC rat, Cu was markedly accumulated, which resulted in an abnormal induction of MT. When the Cu in the liver can no longer bind to the MT protein, Cu toxicosis, or hepatitis, may occur in the LEC rat. Furthermore, in hepatitis the induction of MT is reduced (unpublished data). Eventually, release of Cu from the MT may be the trigger for hepatocellular carcinomas.
In the intestinal mucosa of LEC rats, the Cu concentration in the cytosol fraction (7.4 ± 2.4 μg/g protein) was approximately 1/2 of that in Fischer rats (14.3 ± 6.6 μg/g protein), although Cu concentrations in the intestine were almost the same in the two strains (table 1). In the LEC rat, at least, Cu absorption may not be enhanced in the gastrointestinal tract. Further studies are needed to clarify these phenomena.
The high concentration of Zn in the LEC liver (table 1) may be due to MT induction. However, the increase was not much more pronounced than could be anticipated from the concentration of hepatic Cu (table 1). This result suggests that Cu displaces Zn from MT, which results in an increase of the Cu/Zn ratio in MT (table 2). The distribution of Zn was very similar to that in animals treated with a high dose of Cd[17].
A defect of Cp will influence Fe metabolism. In Wilson's disease, which has low Cp activity, an increase of Fe is found in the liver and spleen, but serum Fe decreases[18]. Furthermore, in Cu-deficient animals with low Cp activity, the same change of Fe is observed[19]. Although the LEC rat had reduced Cp activity, the Fe metabolism was different from that of Wilson's disease. Now we are carrying out further experiments to determine whether the sudden hepatitis observed in LEC rats three to four months after birth is related to the abnormal distribution of Fe, as well as that of Cu.

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Reversible juvenile hormone inhibition of ecdysteroid and juvenile hormone synthesis by the ring gland of *Drosophila melanogaster*

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Abstract. Juvenile hormone bisepoxide (JHB_3) and juvenile hormone III (JH III) both inhibited the in vitro production of ecdysteroids by ring glands and brain-ring gland complexes from third instar post-feeding larvae of *Drosophila melanogaster* in a reversible manner, although JHB_3 had greater efficacy. The JH III and JHB_3 precursor, methyl farnesoate, did not affect ecdysteroid production. The in vitro synthesis of total detectable JH (JHB_3 + JH III + methyl farnesoate) by the corpus allatum portion of the isolated ring gland was also inhibited reversibly in the presence of exogenous JHB_3 and JH III, but not by methyl farnesoate. These data indicating negative feedback are in agreement with the accepted dogma of endocrine gland regulation.
Key words. Juvenile hormone; corpus allatum; prothoracic gland; dipteran endocrinology.

The interplay of ecdysteroids and juvenile hormones (JH) in larval insects serves to orchestrate the progression from one developmental stage to the next, with ecdysteroids regulating the onset and timing of the molting cycle and JH regulating the quality of the molt[1]. Ecdysteroids are produced by the prothoracic glands under the control of the brain via prothoracicotropic hormone (PTTH)[1]. In dipteran larvae, these glands are fused with the corpus allatum, the site of JH synthesis, and the corpus cardiacum, to form a unique composite endocrine organ, the ring gland. As part of an ongoing project on the endocrinology of *Drosophila melanogaster* that will provide a base for genetic analysis, we investigated the ability of juvenile hormone bisepoxide (JHB_3), the presumed major JH of higher flies, and its putative precursor JH III[2], to modulate the production of both ecdysteroids and JHs in vitro by brain-ring gland complexes and isolated ring glands from third instar post-feeding larvae.

Materials and methods

Animals. Wild type *Drosophila melanogaster* (Canton-S strain) were maintained at 25 °C under a 12L:12D photoperiod and fed on *Drosophila* medium (Carolina Biological Co.) supplemented with dried bakers yeast. They were staged according to both wandering behavior and salivary gland morphology[3]. Mid postfeeding stage larvae were used in all experiments.

Chemicals. Methyl farnesoate and JHB_3[2] were synthesized by Drs F. Baker and D. Schooley (Sandoz Crop Protection Co.) while JH III was purchased from Sigma. The JH esterase inhibitor octyl-1,1,1-trifluoropropanone (OTFP)[4] was a gift from Dr M. Roe (North Carolina State University) and was added to all incubations to a final concentration of 0.5 μM. The JHs were stored at −80 °C in either acetonitrile (JHB_3 and JH III) or hexane (methyl farnesoate) at a concentration of 1 mg · ml^{-1} and as required, aliquots were dried down under N_2 in siliconized microfuge tubes at room temper-