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Isolation of a Lipolytic Factor (Toxohormone-L) from Ascites Fluid of Patients with Hepatoma and its Effect on Feeding Behavior*

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Abstract—*The ascites fluids from patients with hepatoma or ovarian tumor and the pleural fluid from patients with malignant lymphoma elicited fatty acid release in slices of rat adipose tissue* in vitro. *The lipolytic factor, named toxohormone-L, was isolated from the ascites fluid of patients with hepatoma. The isolated preparation gave a single band on both disc gel electrophoresis and sodium dodecyl sulfate* (SDS)*-acrylamide gel electrophoresis in the presence of β-mercaptoethanol. Its molecular weight was determined to be* 70,000–75,000 *and* 65,200 *by SDS-acrylamide gel electrophoresis and analytical ultracentrifugation respectively. Injection of toxohormone-L into the lateral ventricle of rats significantly suppressed food and water intakes. There was at least a* 5*-hr delay between its injection and the appearance of its suppressive effect.*

INTRODUCTION

TUMOR-BEARING animals and patients with various neoplasms frequently show a striking depletion of body lipid [1, 2]. This depletion could be related to growth of the tumor and could contribute to debilitation of the host. However, the physiological mechanism involved in this effect of the tumor on the host is unknown.

Significant increases of plasma free fatty acid (FFA) [3, 4] and lipid [5, 6] are frequently observed in animals and patients with neoplastic diseases. These increases could be secondary to increased mobilization of FFA from the host's adipose tissue by a lipolytic substance, and there are reports on the existence of a lipolytic substance in the ascites fluid of rats with Walker 256 carcinoma [7], in the serum of mice with advanced lymphoma [8], in a tumor extract [8] and in the culture medium of lymphoma cells [8].

Previously, we reported that the body triglyceride content decreased during growth of sarcoma 180 in mice and suggested that this reduction could in part be related to the appearance of a lipolytic factor in the ascites fluid [9]. This lipolytic factor, which was also present in the ascited fluids of patients with hepatoma or Grawitz's tumor, was named 'toxohormone-L'. It was not found in non-cancerous ascites fluids. We suggested that lipid depletion in tumor hosts may be explained in part by the action of toxohormone-L on the adipose tissue.

Anorexia is also a common symptom in cancer patients and causes progressive weight loss and progressive cachexia. Peptides, oligonucleotides, and other small metabolites or circulating substances were proposed to be produced by the cancer tissue or to be responsible for the anorexia [10, 11]. However, these substances have not yet been isolated in homogeneous states.

Various lipolytic substances have been isolated from the urine of starving animals and humans and from the urine of patients with widespread malignant neoplasms [12–14], and these lipolytic substances were also reported to cause anorexia [15–18].

The question arises of whether toxohormone-L affects the feeding behaviour directly. This paper reports the isolation of toxohormone-L from the ascites fluid of patients with hepatoma and demonstrates that toxohormone-L suppressed both feeding and drinking of normal rats.

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MATERIALS AND METHODS

Materials

Bovine albumin (fraction V from bovine plasma, Armour Pharmaceutical Co., Phoenix, AZ), DEAE-cellulose DE-52 (Whatman Ltd., Springfield Mill, Maidstone, Kent, U.K.), Blue Sepharose CL-6B, Polybuffer 74 and Polybuffer exchanger PBE-94 for chromatofocusing (Pharmacia Fine Chemicals, Uppsala, Sweden) and molecular weight standards (Mann Research Laboratories, New York) were used.

Animals

Male Wistar strain rats, weighing 150–180 g, were used. They were given standard laboratory diet and water *ad libitum*.

Collection of ascites fluid

Ascites fluids for isolation of toxohormone-L were obtained at autopsy from six patients with hepatoma. The length of the time between death and obtaining the ascites fluid was 2–3 hr. The ascites fluid was centrifuged at 1000 *g* for 10 min at 4°C to remove cell debris and the resultant supernatant was stored at −20°C until use.

Estimation of lipolytic activity of toxohormone-L

Lipolytic activity was measured as described previously [9]. Male rats were killed by a blow on the head, and their epididymal adipose tissue was removed quickly and cut up with scissors. Then 100 mg of minced adipose tissue was incubated in a glass-stoppered test tube for 2 hr at 37°C with 1 ml of Krebs–Ringer bicarbonate buffer (pH 7.4) containing 2.5% bovine albumin and 0.1 mM $CaCl_2$ in the presence of toxohormone-L. After incubation, FFA was extracted and titrated with NaOH as described by Dole [19]. The value for released FFA was corrected for the FFA content at 0 time. A control without toxohormone-L was subjected to the same treatment. The net lipolytic activity induced by toxohormone-L was calculated by subtracting the FFA content of the control. One unit of toxohormone-L activity was defined as the amount causing release of 0.1 μEq of FFA in 2 hr.

Assay of anorexigenic effect of toxohormone-L

Adult male rats, weighing 270–300 g, were housed in individual cages. At 10–14 days before experiments a 23-gauge stainless steel cannula (10.0 mm length) was implanted in the lateral ventricle, according to the stereotaxic coordinates of Pellegrino *et al.* (1.5 mm lateral and 0.4 mm posterior to the bregma and 3.5 mm from the top of the skull) [20]. The cannula was provided with a mandril to prevent its obstruction. For 3 days after the operation rats were allowed free access to pellet food and water, and then powdered laboratory diet was substituted for the pellet food. Restricted feeding for 2 hr (14:00–16:00) per day was started from day 7 after the operation. Microinjection of isotonic saline (0.15 M), toxohormone-L or bovine albumin was performed as follows: the materials were prepared in saline and injected in a volume of 10 μl (for 90 sec) into the lateral ventricle. The same volume of saline or bovine albumin was injected as a control. Immediately after injection of the test material a feeding cup was introduced into the cage and a drinking tube was substituted for the water bottle. Then the total amounts of food and water consumed in 2 hr were measured daily for 5 days. Food intake was measured by weighing the feeding cup before and after feeding. Water intake was determined with a calibrated drinking tube. Test materials were administered once a week.

Gel electrophoresis

Disc gel electrophoresis was carried out by the method of Davis [21] on 7.5% acrylamide gel. SDS–acrylamide gel electrophoresis was carried out on 10% acrylamide gel containing 0.1% SDS in the presence of β-mercaptoethanol. Protein was located by staining the gel with Coomassie Brilliant Blue G-250 in 12.5% trichloroacetic acid and destaining with 7% acetic acid.

Sedimentation equilibrium study

Sedimentation equilibrium was performed in a Hitachi 282 analytical ultracentrifuge in 0.1 M potassium phosphate buffer (pH 7.0) at 50,000 rev/min at 26.3°C.

Protein determination

Protein was measured by the method of Lowry *et al.* [22].

Statistics

Data in Figs 5 and 6 were analyzed by 1- and 2-way analyses of variance (by Sheffe's test) and data in Fig. 7 and Table 2 were analyzed by paired *t*-test.

RESULTS

Lipolytic activity in the ascites fluid

The ascites fluid was obtained from patients with hepatoma at autopsy and the lipolytic activity was measured by incubating slices of adipose tissue from normal rats in buffer containing 0.2 ml of the ascites fluid. The activity was 7.0 ± 1.7 ($n = 6$) units/mg protein. Since there is a danger that toxohormone-L might have been altered in some way because of post-mortem, we measured the lipolytic activities in the ascites fluids obtained from living patients with

hepatoma or ovarian tumor. The activities in these ascites fluids, which were 7.0 ± 0.2 ($n = 6$) and 7.3 ± 0.3 ($n = 4$) units/mg protein respectively, were similar to that in the ascites fluid obtained from cancer patients at autopsy. The pleural fluid obtained from patients with malignant lymphoma at autopsy also elicited fatty acid release and its activity was 7.7 ± 1.0 ($n = 4$) units/mg protein.

Isolation of toxohormone-L

Toxohormone-L was isolated from the ascites fluid of patients with hepatoma. When the ascites fluid was fractionated with ammonium sulfate, the lipolytic activity was recovered in the fraction precipitated at 40–70% saturation. A chromatogram of this fraction on a DEAE-cellulose column is shown in Fig. 1. The lipolytic activity was eluted with 0.15 M ammonium bicarbonate buffer (pH 8.2) and 8.7% of the applied protein was recovered in this fraction. The active fraction eluted from the DEAE-cellulose column was subjected to a Blue Sepharose column in order to eliminate serum albumin (Fig. 2). The lipolytic activity was passed through the column. Next, the fraction passed through above column was chromatographed on a chromatofocusing column. As shown in Fig. 3(a), four main peaks were eluted and toxohormone-L activity showed an isoelectric point of 4.7 to 4.8. This fraction was rechromatographed on the same column (Fig. 3b). Table 1 summarizes the purification procedure. In this way, 5.6% of the activity of

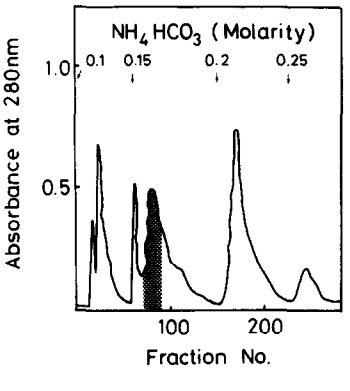

Fig. 1. DEAE-cellulose column chromatography of toxohormone-L in the ascites fluid of patients with hepatoma. The ascites fluid was fractionated with ammonium sulfate. The fraction precipitated at 40–70% saturation was dissolved in and dialyzed against 0.1 M *ammonium bicarbonate buffer (pH* 8.2*). The inner solution was then applied to a DEAE-cellulose column equilibrated with the same buffer. Material was eluted with the same buffer and then stepwise with ammonium bicarbonate buffer of pH* 8.2 *at concentrations of* 0.15 M, 0.2 M *and* 0.25 M. *The effluent from the column was collected in* 10-*ml fractions at a flow rate of* 30 ml/hr, *and the absorbance of each fraction at* 280 nm *was measured. The shaded area indicates fractions used for chromatography on a Blue Sepharose column. Column size:* 2.7 × 16.5 cm. ———, A_{280}.

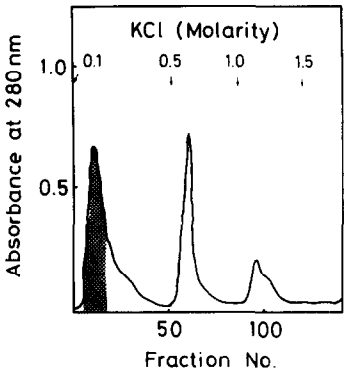

Fig. 2. Blue Sepharose column chromatography of toxohormone-L from the ascites fluid of patients with hepatoma. The shaded fractions eluted with 0.15 M *ammonium bicarbonate buffer from a DEAE-cellulose column (Fig.* 1*) were lyophilized and dissolved in and dialyzed against* 0.05 M *Tris*–HCl *buffer (pH* 7.0*) containing* 0.1 M KCl. *The inner solution was then applied to a Blue Sepharose column equilibrated with the same buffer. Material was eluted with the same buffer and then stepwise with Tris*–HCl *buffer (pH* 7.0*) containing* KCl (0.5 M, 1.0 M, 1.5 M). *The effluent from the column was collected in 5-ml fractions at a flow rate of* 30 ml/hr, *and the absorbance of each fraction at* 280 nm *was measured. The shaded area indicates fractions used for chromatography on a chromatofocusing column. Column size:* 1.7 × 8.5 cm. ———, A_{280}.

toxohormone-L was recovered from the ascites fluid. Figure 4 (left and center) shows the patterns on gel electrophoresis of toxohormone-L eluted from the chromatofocusing column. The protein moved as a single band on disc gel electrophoresis and SDS–acrylamide gel electrophoresis in the presence of β-mercaptoethanol. The mobility on gel of toxohormone-L from the ascites fluid of patients with hepatoma (Fig. 4, left) appeared very similar to that of toxohormone-L from the ascites fluid of mice with sarcoma 180 (Fig. 4, right).

Toxohormone-L from the ascites fluid of patients with hepatoma had a mobility corresponding to a molecular weight of 70,000–75,000 on SDS–acrylamide gel electrophoresis in the presence of β-mercaptoethanol. On sedimentation analysis toxohormone-L sedimented as a single peak with a sedimentation coefficient of 4.81S. The average molecular weight calculated from the sedimentation equilibrium data was 65,200. Thus toxohormone-L eluted from the chromatofocusing column was at least 95% pure.

Anorexigenic effect of toxohormone-L

The anorexigenic effect of toxohormone-L was studied in a group of seven rats that had been trained to ingest all their food in a 2-hr period each day. Injection of toxohormone-L into the lateral ventricle resulted in significant suppression of food intake [doses, $F(3,24) = 2.70$, $P < 0.05$; days, $F(5,120) = 16.65$, $P < 0.01$] (Fig. 5). For

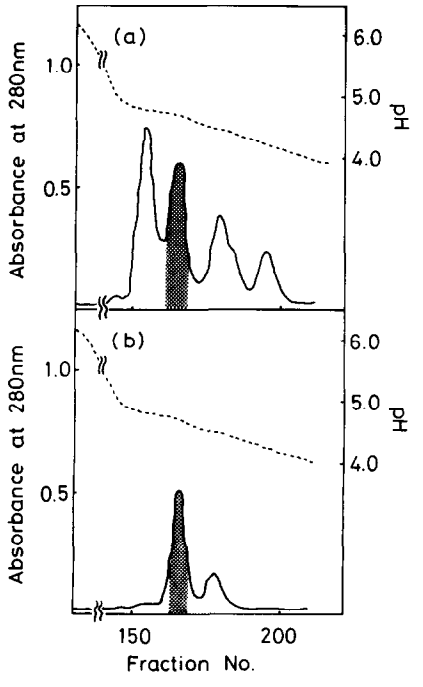

Fig. 3. Chromatofocusing column chromatography of toxohormone-L from the ascites fluid of patients with hepatoma. (a) The fractions passed through a Blue Sepharose column were dialyzed against deionized water and lyophilized. The material was dissolved in 0.025 M *histidine*–HCl *buffer (pH* 6.2) *and applied to a chromatofocusing column equilibrated with the same buffer and eluted with Polybuffer* 74–HCl *buffer (pH* 4.0). *The dilution factor of Polybuffer* 74 *was* 1:8. *Fractions of* 1.3 ml *were collected at a flow rate of* 1.0 ml/min. *To each fraction solid ammonium sulfate was added to* 80% *saturation and the mixture was stood for* 1 hr. *The precipitate was collected by centrifugation and washed twice with saturated ammonium sulfate solution. Then it was dissolved in and dialyzed against deionized water. Toxohormone-L activity was recovered in the position indicated by shading. (b) The active fraction was rechromatographed on the same column. Column size:* 1.0×25.0 cm. ———, A_{280}. ------, *pH.*

determination of the specificity of the effect of toxohormone-L the effect of bovine albumin was measured. Rats injected with 14.4 μg of bovine albumin exhibited no significant alteration in food or water intake compared with that of rats injected with 10 μl of saline or given no treatment (Figs 5 and 6). Toxohormone-L did not have any effect on food intake after 2 hr but suppressed food intake significantly on days 1 and 2 after its injection, compared with the level in rats injected with bovine albumin ($P<0.01$). At the highest dose used in this experiment, daily food intake was suppressed even on day 3.

Toxohormone-L also reduced water intake significantly in a similar manner [doses, $F(3,24)=3.13, P<0.05$; days, $F(5,120)=9.70\ P<0.01$] (Fig. 6). On day 1 after its injection all three doses of toxohormone-L (3.6, 7.2 and 14.4 μg) suppressed water intake compared with that of rats injected with bovine albumin ($P<0.01$). The highest dose of toxohormone-L suppressed water intake even within 2 hr after its injection ($P<0.05$) and caused prolonged suppression.

The amounts of food consumed at the indicated times on day 1 after injection of toxohormone-L were compared with those on day 0 (Fig. 7). A small but significant decrease in the amount of food consumed was observed 15 min after food presentation (day 0, 4.4 ± 0.2 g; day 1, 3.9 ± 0.2 g, $P<0.05$). The rate of food consumption on day 1 then decreased with time compared with that on day 0.

For determination of the latency of development of the suppressive effect of toxohormone-L, 7.2 μg of toxohormone-L was injected into the lateral ventricle 2.5 and 5 hr before giving food. As shown in Table 2, reduction of food and water intakes was observed only when the injection was given 5 hr before feeding time.

DISCUSSION

Previously, we demonstrated the presence of toxohormone-L in cancerous ascites fluids and purified it from the ascites fluid of mice with sarcoma 180 [9]. It was found to be an acidic protein with an isoelectric point of 4.7 and a molecular weight of about 75,000. In the present experiment we estimated the molecular weight of toxohormone-L isolated from the ascites fluid of patients with hepatoma as 70,000–75,000 by gel electrophoresis and 65,200 by ultracentrifugation.

Table 1. Isolation of toxohormone-L from the ascites fluid of patients with hepatoma

Isolation step	Total protein (mg)	Total activity (units)	Yield (%)	Specific activity (units/mg)	Purification (fold)
Ascites fluid	1125.0	10800	100.0	9.6	1.0
40–70% $(NH_4)_2SO_4$, ppt	138.7	8800	81.5	63.4	6.6
DEAE-cellulose column	12.0	3325	30.8	277.1	28.9
Blue Sepharose column	8.2	2531	23.4	308.4	32.2
1st chromatofocusing column	2.6	1144	10.6	440.0	45.8
2nd chromatofocusing column	0.8	604	5.6	755.0	78.6

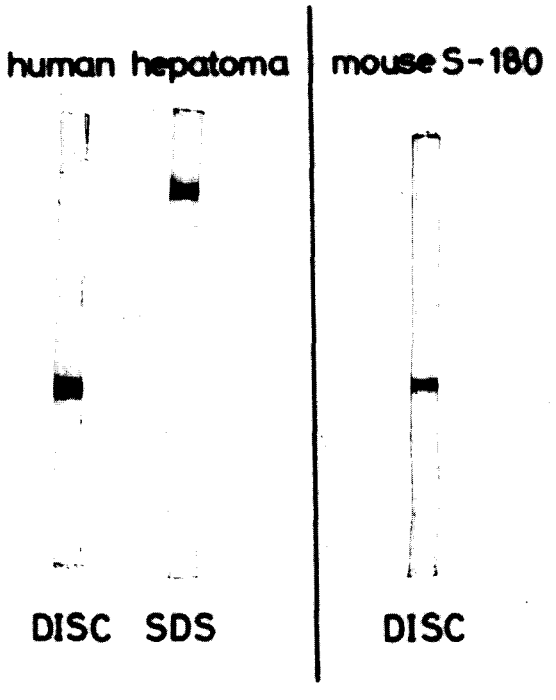

Fig. 4. *Polyacrylamide gel electrophoresis of isolated toxohormone-L. Left: disc gel electrophoresis of toxohormone-L from the ascites fluid of patients with hepatoma. Center: SDS-acrylamide gel electrophoresis of toxohormone-L from the ascites fluid of patients with hepatoma in the presence of β-mercaptoethanol. Right: disc gel electrophoresis of toxohormone-L from the ascites fluid of mice with sarcoma* 180.

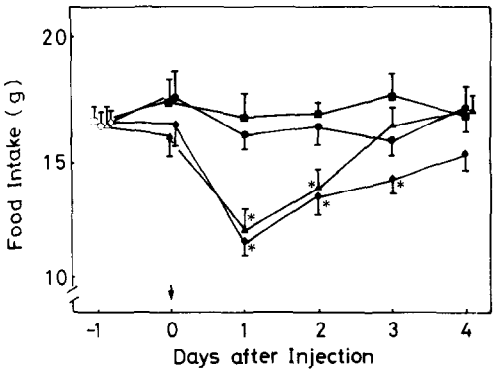

Fig. 5. Effect of intraventricular injection of toxohormone-L on food intake. The total amounts of food consumed in 2 hr *were measured daily. Points and bars are means* ± *S.E.* (n = 7 *rats). The arrow shows the time of injection of toxohormone-L into the lateral ventricle.* □,○,△,◇, *saline injection;* ■, 14.4 μg *bovine albumin;* •, 3.6 μg *toxohormone-L;* ▲, 7.2 μg *toxohormone-L;* ◆, 14.4 μg *toxohormone-L.* *, $P < 0.01$ *(compared with bovine albumin).*

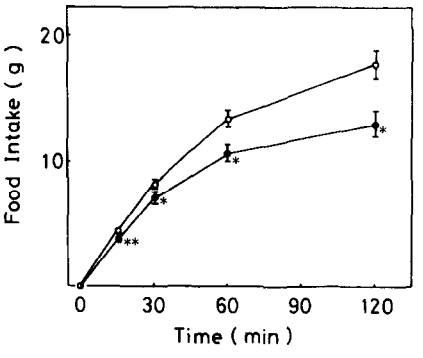

Fig. 7. Time course of suppression of food intake by toxohormone-L. 7.2 μg *toxohormone-L was injected into the lateral ventricle. The amounts of food consumed at the indicated times were measured on days* 0 (○) *and* 1 (•) *after its injection. Significant difference from the value on day* 0: *, $P < 0.01$; **, $P < 0.05$.

Nakahara and Fukuoka [23] first extracted a factor depressing liver catalase activity from tumor tissues and named it 'toxohormone'. It was thought to be a protein substance that produced changes, such as depression of liver catalase activity and decrease in the serum iron level, that are associated with tumor bearing. Fujii *et al.* [24] showed that decrease in plasma iron was due to a different substance from that decreasing liver catalase activity and proposed that toxohormone is not a single substance but a complex of several components. However, toxohormone has not yet been isolated in a chemically pure state. Therefore we examined the effect of our preparation of toxohormone-L on liver catalase activity and the serum iron level, finding that intraperitoneal injection of toxohormone-L had no effect on either (data not shown). Hence toxohormone-L seems to be a different substance from the factor depressing liver catalase and that decreasing the serum iron level. Toxohormone-L also did not cause increase in [^{14}C]-thymidine incorporation into liver DNA (data not shown).

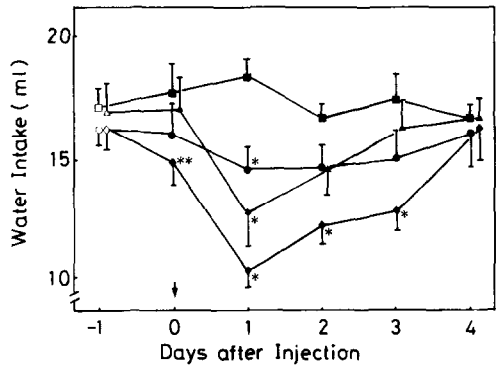

Fig. 6. Effect of intraventricular injection of toxohormone-L on water intake. The total amounts of water consumed in 2 hr *were measured daily. Points and bars are means* ± *S.E.* (n = 7 *rats). The arrow shows the time of injection of toxohormone-L into the lateral ventricle.* □,○,△, ◇, *saline injection;* ■, 14.4 μg *bovine albumin;* •, 3.6 μg *toxohormone-L;* ▲, 7.2 μg *toxohormone-L;* ◆, 14.4 μg *toxohormone-L.* *, $P < 0.01$; **, $P < 0.05$ *(compared with bovine albumin).*

Anorexia and subsequent weight loss are often seen in patients with neoplastic diseases [25]. Their cause is unknown, although many possible mechanisms have been suggested [11, 26–29]. One possible explanation for the anorexia is the production of some anorexigenic substance by the tumor [10, 11].

In the present study, injection of toxohormone-L into the lateral ventricle resulted in significant decreases in food and water intakes (Figs 5 and 6). An additional experiment with a different feeding time (09:00–11:00) was carried out to determine whether injection of toxohormone-L into the lateral ventricle merely altered the diurnal periodicity of the feeding pattern. Injection of 7.2 μg toxohormone-L resulted in significant suppression of food and water intakes in the same manner, as mentioned in the results (data not shown). These results suggest that toxohormone-L also has an anorexigenic action and that it might contribute to weight loss in patients with cancer and tumor-bearing animals.

We observed that almost all rats began to feed within 10 sec after introducing food into the cage, regardless of whether they had received an injection of toxohormone-L, but that they ate less because of a reduced eating rate (Fig. 7). No behavioral toxicities of toxohormone-L such as ataxia, sedation and muscle relaxation were noticed during the test periods.

Table 2. Latency in development of suppression of food and water intakes by toxohormone-L

Administration	Before giving food (hr)	Food intake (g)	Water intake (ml)
Saline	0	17.1 ± 0.6	17.7 ± 0.9
Toxohormone-L (7.2 μg)	0	17.6 ± 1.1	16.9 ± 1.3
	2.5	16.5 ± 0.6	16.7 ± 0.8
	5.0	14.1 ± 0.4*	13.9 ± 0.9*
	24.0	10.7 ± 0.9*	10.6 ± 0.7*
Bovine albumin (7.2 μg)	0	17.1 ± 0.9	17.0 ± 1.3
	2.5	17.4 ± 1.0	17.1 ± 0.8
	5.0	17.1 ± 0.6	17.9 ± 1.1
	24.0	16.9 ± 1.0	17.2 ± 1.0

7.2 μg of toxohormone-L was injected into the lateral ventricle 2.5 and 5.0 hr before giving food. The total amounts of food and water consumed in 2 hr were measured. Details are as described in Materials and Methods.
*Significant difference from the value for rats injected with bovine albumin; $P < 0.01$.

Previously, we reported that when toxohormone-L from the ascites fluid of mice with sarcoma 180 was digested with trypsin and then the digest was dialyzed against deionized water, 30% of its lipolytic activity was recovered in the outer solution [9]. From this finding, the delay in the onset of the anorexigenic action of toxohormone-L and the persistence of its action might be explained by slow liberation of a biologically active fragment from toxohormone-L in the brain. However, whether toxohormone-L and its active fragment can cross the blood–brain barrier is still unknown in the present study. In addition, the possibility that another anorexigenic substance is produced or released by the host in the response to some signal from toxohormone-L cannot be excluded.

The mechanism by which toxohormone-L suppressed food intake is now the subject of thorough investigation.

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