IMPAIRMENTS OF GLYCOGEN AND DRUG METADOLISMS BY CHLOROPEPTIDE, A HEPATOTOXIC MYCOTOXIN OF *PENICILLIUM ISLANDICUM* SOPP¹⁾

Yoshio UENO and Teruo ITO

*Department of Microbial Chemistry and Toxicology, Faculty of Pharmaceutical
Sciences, Tokyo University of Science, Ichigaya, Shinjuku-ku, Tokyo 162, Japan*

Ikuko UENO

*Department of Carcinogenicity and Cancer Susceptibility, Institute of Medical
Sciences, University of Tokyo, Shirogane, Minato-ku, Tokyo 108, Japan*

Received December 15, 1977

Abstract.....Administration of chloropeptide, a hepatotoxic cyclic pentapeptide of *Penicillium islandicum* Sopp, caused a marked decline in the hepatic glycogen synthetase activity with concomitant increase in NADP-dependent G-6-P dehydrogenase activity. The microsomal mixed function oxygenases were also reduced in the liver of rats following chloropeptide treatment.

Key words : chlorine-containing peptide (chloropeptide), *Penicillium islandicum* Sopp, glycogen synthetase, hepatotoxin, mixed function oxygenase

INTRODUCTION

During the course of toxicological survey on metabolites of *Penicillium islandicum* Sopp, Tatsuno *et al.* (1955) isolated a cyclic pentapeptide from culture filtrates of the fungus, and from the evidence that the peptide contains two chlorine atoms, they named the compound as "chlorine-containing peptide". Uraguchi *et al.* carried out toxicological researches on the peptide and demonstrated that this peptide is highly hepatotoxic and carcinogenic to mice and rats. (Uraguchi *et al.*, 1961 and 1972).

Chemical and physical approaches revealed that the molecular formula is $C_{24}H_{31}N_5O_7Cl_2 \cdot 2H_2O$ (M.W. 607) and the amino acid sequence is [L-ser→L-dichloropro→L-α-amino-*n*-butyric acid→L-ser→L-β-phe→]. Two chlorine atoms attach to the pyrrolidine ring of proline in *cis* configuration, and they revised the name to "cyclochlorotine" (Yoshioka *et al.* 1973).

In the preceding paper, the authors examined the acute toxicity of the peptide to

1) Presented in part at the 1st. International Congress of Toxicology, Toronto, Canada, April 3, 1977 (Ueno *et al.*, 1977b) and Jap. Association of Mycotoxicology, Kobe, September 3, 1977 (Ueno *et al.*, 1977c).

2) This study (Yellow Rice Research, Section K) was aided by a grant from the Ministry of Welfare.

連絡先 : 〒162 東京都新宿区市ヶ谷船河原町12番地 東京理科大学薬学部 上野 芳夫

various laboratory animals, and the s.c. LD₅₀ values to mice, rats and guinea-pig were estimated to 0.5 mg/kg. Biochemical approaches demonstrated that the toxin reduced *in vivo* glycogen content in the liver of mice and rats shortly after the administration of peptide (Ueno *et al.* 1977a). In the present paper, the authors aimed to analyze the functional damage of liver of mice and rats by the peptide, and examined the *in vivo* effect on carbohydrate metabolism and related enzyme activities in whole animal level, liver slice level and liver subcellular level. The results revealed that the toxin reduced glycogen synthetase activity and accelerated G-6-P dehydrogenase.

METHODS

Chloropeptide. Crystalline chlorine-containing peptide (abbreviated to Cl-peptide) was isolated from the culture filtrate of *P. islandicum* Sopp according to the method previously reported (Ishii and Ueno, 1973). For animal experiments, the toxin was dissolved in 0.9% NaCl solution immediately before use, and a sublethal dose (0.1–0.5 mg/kg) was s.c. administered to mice and rats.

Animals. Male ddYS mice (6 week old) and male Wistar rats (6 week old) were obtained from the Agricultural Cooperative for Experimental Animals (Hamamatsu). The animals were housed in air-conditioned quarters and fed standard commercial diets and water ad libitum. In some experiments, the animals were fasted overnight.

Measurements. For determination of liver glycogen, 100–200 mg of liver samples and liver slices were mixed with 2 ml of 1.0 N KOH and incubated at 37°C overnight, and glycogen was precipitated by addition of 3 volumes of ethanol. After washing the precipitate by water-ethanol, glycogen was determined by anthrone method (Hassid *et al.*, 1957). Protein in subcellular fractions was determined by a modification of the procedure of Layne (1957). Lactic acid and inorganic phosphate (Pi) were determined by the methods of Barker (1957) and Fiske-SubbaRow (Leloir *et al.*, 1957), respectively. The activities of aminopyrine demethylase and aniline hydroxylase were determined by estimating formaldehyde (Nash, 1953) and p-aminophenol (PAP) (Imai *et al.*, 1966).

Fractionation of liver tissues. For the carbohydrate metabolism, liver tissue was washed with cold isotonic NaCl solution and homogenized in 9 volumes of 0.25 M sucrose solution in a glass-Teflon homogenizer. Post mitochondria, post lysosomes and microsomes were obtained by stepwise centrifugation at 9,000 g × 20 min, 24,000 g × 40 min, and 105,000 g × 90 min, respectively. The final supernatant fraction was referred to as cytosol. For the drug metabolism, liver tissue was homogenized in 3 volumes of 1.15% KCl solution, and 12,000 g × 15 min supernatant was centrifuged at 105,000 g for 90 min. The pellets were suspended into 1.15% KCl in concentration of microsomes equivalent to one g liver per ml.

Radioisotopes and counting. U-¹⁴C-glucose (5.0 mCi/mM), 1-¹⁴C-glucose (25 mCi/mM), 2-¹⁴C-acetic acid (20.0 mCi/mM), and ¹⁴C-casamino acid (22 mCi/mg) were purchased from Daiichi Chemicals LTD. (Tokyo). Radioactivity was determined by using gas-flow counter.

RESULTS

1) *Experiments with whole animals*

Since the peptide caused rapid decrease of hepatic glycogen in mice and rats as reported in the preceding paper (Ueno *et al.* 1977a), the glycogen synthetic ability was examined in whole animal level. Male mice were starved overnight to reduce an endogenous glycogen content in the liver, and they were administered with glucose solution intraperitoneally. As shown in Fig. 1, in the control mice, the hepatic glycogen increased quickly and reached to maximum level 20 min after the administration. While, in the mice administered with 0.5 mg/kg of Cl-peptide 30 min prior to the glucose administration, no increment of hepatic glycogen was observed. Similar results were obtained in rats which received s.c. the peptide in doses of 0.1–0.5 mg/kg (data are not presented).

With an aim to confirm the depressed ability of glycogen synthesis *in vivo*, the incorporations of labelled glucose and acetic acid into hepatic glycogen were examined in the control and Cl-peptide treated mice. Mice, starved overnight, were received i.p. U-¹⁴C-glucose or 2-¹⁴C-acetic acid, and the radioactivity in hepatic glycogen fraction was counted. As shown in Fig. 2, in the normal mice, the radioactivity increased up to one

Fig. 1. Inhibition of glycogen synthesis *in vivo* by chloropeptide in mice. Mice, starved for 16 hours, were administered with 0.1 ml/10 g body weight of 20% glucose solution at 0 time. ●—● control; ●-----● Cl-peptide was s.c. administered in dose of 0.5 mg/kg 30 min prior to the administration of glucose. Each point is an average of 5 mice.

Fig. 2. Inhibitory effect of chloro peptide to the incorporations of labelled glucose and acetate into liver glycogen *in vivo*. $0.1 \mu\text{Ci}/10\text{g}$ of ^{14}C -U-glucose (A) or ^{14}C -2-acetate was i.p. administered to mice. $\bigcirc$ — $\bigcirc$ control; $\bullet$ — $\bullet$ mice received s.c. 0.5 mg/kg of Cl-peptide 30 min prior to the labelled compounds.

Fig. 3. Effect of chloro peptide to the incorporation of ^{14}C -amino acids into liver protein and fat. $0.1 \mu\text{Ci}/10\text{g}$ of ^{14}C -casamino acid was i.p. administered to mice at 0 time as indicated in the figure. $\bigcirc$ — $\bigcirc$ control; $\bullet$ — $\bullet$ mice received 0.5 mg/kg of Cl-peptide s.c. 30 min prior to the labelled amino acids.

Metabolic impairments by chloropeptide

Table 1. Microsomal drug-metabolizing enzymes in the liver of rats administered chloropeptide.

Time after Cl-peptide ^{a)} (hours)	Wet weight of liver (g)	Microsomal protein (mg/g liver)	Aminopyrine demethylase ^{b)} (nmoles HCHO/mg protein/30 min)	Aniline hydroxylase ^{b)} (nmoles PAP/mg protein/30 min)
0	5.9	20.7	108.7	13.5
	5.7(5.9) ^{c)}	21.0(20.4)	110.7(108.8)	15.7(14.5)
	6.2	19.4	107.0	14.4
1	8.1	16.9	93.3	12.2
	8.6(8.2)	14.6(16.3)	121.3(104.1)	12.2(12.1)
	8.0	16.6	97.7	11.9
3	7.7	22.7	90.3	10.7
	8.0(7.4)	17.4(20.3)	87.3(84.7)	10.9(10.3)
	6.6	20.9	76.7	9.4
6	7.6	26.1	60.7	7.2
	6.4(7.0)	21.7(23.2)	90.0(77.2)	10.1(8.8)
	6.9	21.7	81.0	9.1
20 ^{d)}	6.7	13.5	83.3	8.2
	6.6(6.6)	15.4(14.5)	69.7(76.5)	6.4(7.3)
	—	—	—	—

a) Three rats were s.c. administered with 0.25 mg/kg of Cl-peptide.

b) 0.3 M phosphate buffer (pH 7.4) 0.4 ml, 0.2 M MgCl₂ 0.1 ml, 0.1 M nicotinamide 0.2 ml, 50 mM glucose-6-phosphate 0.4 ml, 2 mM NADP 0.3 ml, glucose-6-phosphate dehydrogenase 1.2 I.U., 20 mM aminopyrine or aniline 0.1 ml, and microsomes equivalent to 0.4 g liver, in total volume of 2.0 ml, were incubated at 37°C for 30 min. Reactions were stopped by adding 1.0 ml of 30% PCA (aminopyrine demethylase) or 20% TCA (aniline hydroxylase).

c) Parenthesis indicates an average of three estimations.

d) One out of three rats died 20 hours after Cl-peptide.

hour after the injection of labelled precursors. While, in the peptide-treated mice which received s.c. 0.5 mg/kg of Cl-peptide 30 min prior to the labelled compounds, the incorporation into glycogen was markedly depressed.

As for the synthesis of protein and fat in the liver, the incorporation of ¹⁴C-amino acids into hepatic protein and lipid fractions was examined. As shown in Fig. 3, the incorporation of ¹⁴C-amino acid into protein was not affected by Cl-peptide, while the incorporation into lipid fraction was accelerated by 120–130% of the control.

Further experiments with microsomes derived from the liver of control and Cl-peptide-treated rats revealed that the microsomal drug-metabolizing enzymes such as aminopyrine demethylase and aniline hydroxylase were decreased by 50–70% of the control several hours after the s.c. injection of 0.25 mg/kg of Cl-peptide, as summarized in Table 1.

These *in vivo* experiments revealed that the peptide caused a marked depression of glycogen-synthesizing system in the early stage and drug metabolizing enzyme system in the later stage of intoxication.

2) Experiments with liver slices

As a step to disclose the mechanism of impairment of glycogen metabolism, experi-

Fig. 4. Inhibition of glycogen synthesis in the liver slices of rats administered chloropeptide. 70 mmoles KCl, 20 mmoles $MgCl_2$, 10 mmoles $CaCl_2$, 40 mmoles $KHCO_3$, 28 mmoles glucose, and 200 mg of liver slice, in total volume of 2.0 ml, were incubated at 37 C for 60 min. (A) Rats, starved overnight, were received s.c. 0.5 mg/kg of Cl-peptide. (B) Rats, starved overnight, were sacrificed 3 hours after s.c. administration of various doses of Cl-peptide.

ments were carried out with slices of liver derived from the control and Cl-peptide-treated rats. Preliminary experiments revealed that liver slices derived from the control rats which were starved overnight synthesized glycogen from glucose in the presence of KCl, $MgCl_2$, $CaCl_2$ and $KHCO_3$, and the net increase was proportional to the incubation time up to 60 min. Therefore, the authors examined the effect of Cl-peptide to the synthetic ability of hepatic glycogen in the function of the time of Cl-peptide treatment and the dose.

Fig. 5. Acceleration of the decarboxylation of 1-¹⁴C-glucose in liver slices of rats received chloropeptide. 5 ml Locke-Ringer's solution, 200 mg liver slices, and 0.5 μ Ci 1-¹⁴C-glucose, were incubated at 37 C for 120 min under shaking. Rats, starved overnight, were received 0.5 mg/kg of Cl-peptide and sacrificed at the time indicated in the figure.

As shown in Fig. 4, the slices derived from the normal rat liver accumulated glycogen in 10-15 μ g/100 mg slices. While, in the liver slices derived from rats received s.c. 0.5 mg/kg of Cl-peptide, a marked reduction in glycogen accumulation was observed 0.5 hour after the peptide (Fig. 4-A), and this reduction was dose-dependent (Fig. 4-B).

Noticeable results with the hepatic slices were the acceleration of decarboxylation of 1-¹⁴C-glucose by peptide treatment. When the normal liver slices were incubated in Locke-Ringer's solution, 1-¹⁴C-glucose was decarboxylated into ¹⁴CO₂, and this decarboxylation was proportional to the incubation time up to 150 min. While, with the liver slices derived from the peptide-treated rats, as shown in Fig. 5, the formation of ¹⁴CO₂ from 1-¹⁴C-glucose was enhanced in proportion to the time of peptide treatment, and the increase was reached to 300% of the control 5 hours after Cl-peptide. The acceleration of decarboxylation was specific to 1-¹⁴C-glucose, since no marked change was observed with 6-¹⁴C-glucose (data are not presented).

The metabolism of ^{14}C -acetic acid and ^{14}C -amino acids in the liver slices derived from the Cl-peptide treated rats was compared with that of the control slices. As shown in Fig. 6, the degradation of 2- ^{14}C -acetic acid into $^{14}\text{CO}_2$ was slightly reduced (Fig. 6-A), while, the incorporation into lipid fraction was markedly accelerated in the liver slices of Cl-peptide treated rats, and this acceleration was proportional to the time after the toxin treatment (Fig. 6-B). As for the incorporation of ^{14}C -amino acids into protein fraction, no significant difference was observed between the control and the toxin-treated rats (data are not presented).

3) Experiments with subcellular fractions of livers

As described in the above results, the treatment of mice and rats with a sublethal dose of Cl-peptide caused a marked depression of glycogen synthesis and an increment of lipid synthesis, the authors examined several enzyme activities which control the synthesis and degradation of hepatic glycogen and lipid.

Table 2 summarized the data concerning aerobic and anaerobic glycolytic activities of the liver of mice. The activity, expressed as μmoles lactic acid formed/30 min/20 mg liver was estimated in the presence (exogenous) and the absence (endogenous) of hexose-1,6-diphosphate, and the net glycolytic activity was expressed as a difference of the above two estimations.

The data summarized in Table 2, indicated that the aerobic and anaerobic glycolysis

Fig. 6. Metabolism of 2- ^{14}C -acetic acid in the liver slices of rats received chlorocephal. 5 ml Locke-Linger's solution, 200 mg liver slices, and 0.5 μCi 2- ^{14}C -acetic acid, were incubated at 37 C for 120 min. Rats, starved overnight, were s.c. received 0.5 mg/kg of Cl-peptide, and sacrificed at the time indicated in the figure. Each point is an average $\pm$ S.D. of four determinations.

Metabolic impairments by chloropeptide

Table 2. Aerobic and anaerobic glycolysis in the liver of mice administered with chloropeptide.

Time after Cl-peptide (min)	Lactic acid (μ moles/30 min/20 mg liver)					
	aerobic			anaerobic		
	(+)HDP (A)	(-)HDP (B)	(A)-(B)	(+)HDP (A)	(-)HDP (B)	(A)-(B)
0	8.33 $\pm$ 1.09	2.62 $\pm$ 0.91	5.71 $\pm$ 1.98	8.33 $\pm$ 1.22	2.55 $\pm$ 0.10	5.78 $\pm$ 1.05
15	7.28 $\pm$ 1.24	1.98 $\pm$ 0.19	5.30 $\pm$ 0.29	7.26 $\pm$ 1.26	1.96 $\pm$ 0.08	5.30 $\pm$ 1.08
30	8.32 $\pm$ 1.32	1.61 $\pm$ 0.73	6.71 $\pm$ 1.81	8.33 $\pm$ 2.01	1.61 $\pm$ 0.09	6.72 $\pm$ 1.67
60	7.99 $\pm$ 0.02	1.68 $\pm$ 0.10	6.31 $\pm$ 0.24	7.99 $\pm$ 1.16	1.67 $\pm$ 0.08	6.32 $\pm$ 1.16
120	9.23 $\pm$ 1.46	1.27 $\pm$ 0.01	7.96 $\pm$ 1.82	9.56 $\pm$ 1.76	1.27 $\pm$ 0.09	8.29 $\pm$ 1.19

Mice were s.c. administered with 0.5 mg/kg of Cl-peptide.

Aerobic; 80 μ moles phosphate buffer (pH 7.6), 100 μ moles KHCO_3 , 140 μ moles nicotinamide, 52 μ moles pyruvic acid (K), 1 μ mole ATP (K), 0.9 μ mole NAD, 35 μ moles MgCl_2 , 70 μ moles KF, and 0.2 ml of 10% liver homogenate, in total volume of 3.0 ml, were incubated at 37 C for 20 min in the presence (A) or absence (B) of 15 μ moles hexose diphosphate (HDP). Anaerobic; 80 μ moles phosphate buffer (pH 7.6), 75 μ moles KHCO_3 , 120 μ moles nicotinamide, 15 μ moles pyruvate (K), 1 μ mole ATP (K), 20 μ moles MgCl_2 , 30 μ moles KF, and 0.2 ml of 10% liver homogenate, in total volume of 3.0 ml, were incubated in the presence (A) or absence (B) of 15 μ moles of HDP by using Thunberg tubes under reduced pressure.

Each point is an average $\pm$ S.D. of 3-5 mice.

Table 3. Phosphorylase activity of the liver of mice administered chloropeptide.

Time after Cl-peptide (min)	Phosphorylase activity (μ moles Pi/mgprotein)
0	0.200 $\pm$ 0.02
30	0.206 $\pm$ 0.04
60	0.204 $\pm$ 0.02
120	0.198 $\pm$ 0.01

Five mice were s.c. administered with 0.5 mg/kg of Cl-peptide.

Citrate buffer (pH 5.9) 50 μ moles, G-1-P 16 μ moles, AMP 1 μ mole, cystein 40 μ moles, KF 30 μ moles, and 9000 g $\times$ 20 min supernatant 0.2 ml, in total volume of 2.0 ml, were incubated at 37 C for 15 min, and Pi liberated were estimated.

of the liver homogenate of Cl-peptide-treated mice was not different from that of the control mice, either in the absence or presence of hexose-diphosphate.

Table 3 summerized the activity of phosphorylase of post mitochondrial fraction of the livers of control and Cl-peptide treated mice. During two hours after the s.c. injection of 0.5 mg/kg of Cl-peptide, no significant difference was detected between the control and toxin-treated mice.

Similar results were obtained on the activity of G-6-P ase and F-1, 6-P phosphatase, as summerized in Table 4.

Table 4. G-6-P and F-1, 6-P phosphatase activities in the liver of mice administered with chloropeptide.

Time after Cl-peptide (hours)	G-6-P phosphatase (μ moles Pi/mg protein)		F-1, 6-P phosphatase (μ moles Pi/20 min)
	Exp. 1	Exp. 2	
0	3.19 $\pm$ 0.44	3.12 $\pm$ 0.27	1.24 $\pm$ 0.12
0.5		2.66 $\pm$ 0.18	1.22 $\pm$ 0.18
1.0	3.88 $\pm$ 0.55	3.76 $\pm$ 0.24	1.31 $\pm$ 0.27
2.0		2.78 $\pm$ 0.26	1.21 $\pm$ 0.02
3.0	3.35 $\pm$ 0.45		
5.0		3.12 $\pm$ 0.30	

Five mice were s.c. administered with 0.5 mg/kg of Cl-peptide.

G-6-P phosphatase; 60 μ moles maleate buffer (pH 6.5), 10 μ moles G-6-P, 0.1 ml of 9000 g $\times$ 20min supernatant, in total volume of 0.5 ml, were incubated at 37 C for 15 min. F-1, 6-P phosphatase; 20 μ moles borate buffer (pH 6.5), 5 μ moles F-1, 6-P, 5 μ moles MgSO₄, 0.5 μ mole MnCl₂, 5 μ moles cystein, and 0.2 ml of 10% homogenate, in total volume of 1.0 ml, were incubated at 37 C for 20 min.

Table 5. Glycogen synthetase activity in the liver of mice and rats administered chloropeptide.

Species	Starvation (hours)	Cl-peptide (mg/kg s.c.)	Time (hours)	Glycogen synthetase
				m μ moles UDP/mg protein
(A) 24000 g $\times$ 40 min supernatant fraction as enzyme source				
Mice	0	0		646 (100)*
		0.5	3.0	216 (33)
	16	0		161 (100)
		0.5	1.5	97 (60)
Rats	0	0		268 (100)
		0.5	3.0	134 (50)
	16	0		88 (100)
		0.5	1.5	55 (62)
(B) Microsomal fraction as enzyme source				
Mice	0	0		646 (100)
		0.5	3.0	216 (33)
	6	0		277 (100)
		0.25	1.0	170 (61)
		0.25	3.0	143 (52)
		0.25	5.0	88 (31)
Rats	16	0		78 (100)
		0.5	1.5	48 (61)
		0.5	3.0	30 (39)

* Percent activity of the control

100 μ moles Tris-Hce buffer (pH 8.5), 0.12 μ mole EDTA, 0.17 μ mole UDPG, 2.5 μ moles G-6-P, 0.4 mg glycogen, and 0.1 ml enzyme solution, in total volume of 0.45 ml, were incubated at 37 C for 10 min.

Metabolic impairments by chloropeptide

Effects of Cl-peptide on the activity of hepatic glycogen synthetase of mice and rats were summarized in Table 5. As it is well established that the activity in liver tissue is greatly influenced by nutrient conditions, especially of the content of hepatic glycogen, and that the enzyme localizes in the microsomal fraction, the experiments were carried out with both post lysosomal and microsomal fractions of the livers of mice and rats. The activity was compared under well-fed and fasted conditions. UDP liberated was estimated by using pyruvate and pyruvate kinase system, and the activity was expressed as μ moles UDP liberated/mg protein.

As shown in Table 5, the activity of hepatic glycogen synthetase of normal mice was about 650 μ moles UDP/mg protein, and it declined to 300 and 160 μ moles/mg protein when the mice were starved for 6 and 16 hours, respectively. Similarly, the enzyme activity of normal rats was diminished from 270 μ moles/mg protein to 80–90 μ moles/mg protein upon starvation for 16 hours. Administration of Cl-peptide in doses of 0.25–5 mg/kg s.c. caused a rapid and marked decline of the enzyme activity in both 24,000 g $\times$ 40 min supernatant and microsomal fractions of livers of mice and rats. Since the activity of glycogen synthetase was reduced to 60% of the control value 1–1.5 hours after the s.c. injection of Cl-peptide, it is conceivable that this decline is a direct effect of the peptide on synthetic mechanism of glycogen and ruled out the possibility that it is caused by refusal of feed or starvation in animals.

An interesting finding is that the s.c. administration of Cl-peptide to mice and rats caused a rapid and marked elevation of G-6-P dehydrogenase activity in the hepatic microsome and cytosol fractions. As shown in Table 6, mice were s.c. administered with 0.5 mg/kg of Cl-peptide, and the microsomal G-6-P dehydrogenase was assayed following 5.5 hours. Three independent experiments revealed that the enzymatic activity, expressed as μ moles NADP reduced/min/mg protein, was increased by 120–150% of the control

Table 6. G-6-P dehydrogenase activity of the microsomal fraction of liver of mice administered with chloropeptide.

Time after Cl-peptide (hours)	G-6-P dehydrogenase (m μ moles NADPH/min/mg protein)		
	Exp. 1	Exp. 2	Exp. 3
0	14.3 $\pm$ 3.8(100)*	14.5 $\pm$ 1.5(100)	14.8 $\pm$ 0.3(100)
0.5		22.3 $\pm$ 2.3(153)	15.5 $\pm$ 1.2(105)
1.0			17.8 $\pm$ 1.8(120)
1.5	19.7 $\pm$ 2.1(137)	25.3 $\pm$ 3.1(174)	
2.0		30.3 $\pm$ 2.9(223)	
3.0	28.6 $\pm$ 2.2(200)		24.2 $\pm$ 2.7(164)
5.0		26.6 $\pm$ 2.6(183)	
5.5			30.5 $\pm$ 4.5(206)

Mice were s.c. administered with 0.5 mg/kg of Cl-peptide. Each point is an average of five mice.

200 μ moles Tris-HCl buffer (pH 7.4), 30 μ moles MgCl₂, 0.3 μ mole NADP, 10 μ moles G-6-P, and 0.1 ml microsomal fraction, in total volume of 3.0 ml, were incubated at 25 C, and the increase of optical density at 360 m μ was recorded.

value 0.5–1.5 hours after administration, and this value was further increased by 150–200 % of the control 2.0–5.5 hours after the injection of Cl-peptide. Experiments with the hepatic cytosol fraction of mice indicated that the control value of 20 m μ moles/min/mg protein was increased to 25 (0.5 hour), 26 (1.5 hours) and 31 (3.0 hours) after the s.c. administration of Cl-peptide in dose of 0.2 mg/kg. Another experiment with the hepatic cytosol of rats also indicated that the control value of 72 m μ moles NADP reduced/min/mg protein was increased to 101 (1.0 hour) and 124 (3.0 hours) after the s.c. administration of 0.25 mg/kg of Cl-peptide (data not presented in the Table). On the other hand, the activity of isocitrate dehydrogenase in cytosol, which requires also NADP as a cofactor, was remained in constant during three hours after the s.c. administration of 0.2 mg/kg of Cl-peptide to mice; for example, the control value is 69 m μ moles NADP reduced/min/mg protein, and the activity 3 hours after the administration is 72 m μ moles.

DISCUSSION

In the preceding paper (Ueno *et al.*, 1977a), the authors reported that the s.c. administration of Cl-peptide to mice in doses of 0.2–0.8 mg/kg induced a marked decline of the hepatic glycogen. The initial decline was observed within 30 minutes after the administration at the s.c. dose of 0.5 mg/kg of the peptide, and by the 3rd hour the glycogen content in the treated mice had been reduced to nearly 10% of the control value.

In order to find out the cause why the hepatic glycogen content had been reduced shortly after the administration of Cl-peptide, the synthetic and degrading pathways of hepatic glycogen were examined in various levels such as whole animals, liver slices and subcellular fractions of liver tissue.

As for the synthetic system of hepatic glycogen, a single s.c. injection of Cl-peptide to mice in a sublethal dose caused an inhibition of hepatic increment of glycogen in the whole animal level (Figs. 1 and 2). Such inhibition was also demonstrated *in vitro* using by the liver slices derived from the toxin-treated rats (Fig. 4). This finding strongly indicates that the impairment of glycogen synthesis is originated from a functional damage of hepatic cells itself.

Experiments with enzyme preparations of glycogen synthetase showed that the s.c. administration of Cl-peptide induced a marked reduction in the activity of synthetase, both in the 24,000 g $\times$ 40 min supernatant and the microsomal fractions of damaged livers (Table 5).

From these evidences, it is deduced that a rapid onset of synthetase decline is one of the reasons for a disappearance of hepatic glycogen.

As for the degradation system of hepatic glycogen and related substrates, the authors detected no marked alterations in phosphorylase (Table 3), G-6-P and F-1,6-P phosphatases (Table 4), aerobic and anaerobic glycolysis (Table 2) in the liver of mice treated with Cl-peptide.

However, the experiments with slices of liver revealed an accelerated decarboxylation of C-1 of glucose in the liver of the toxin-treated rats (Fig. 5). Actually, experi-

ments with enzyme preparations from hepatic microsomes and cytosol fractions revealed that NADP-dependent G-6-P dehydrogenase was found to be increased. Initial elevation was observed within 30 minutes after the administration of 0.5 mg/kg of the peptide, and by the 3rd to 5th hour the microsomal G-6-P dehydrogenase activity in the treated rats had been increased to 200% of the control value (Table 6).

Therefore, it is presumed that a rapid onset of increment in the microsomal NADP-dependent G-6-P dehydrogenase is also another reason for the decline of hepatic glycogen content following the peptide treatment.

The acceleration of G-6-P dehydrogenase in the hepatic cells resulted in presumably the elevation of NADPH level. Since it is well established that a synthetic reaction of fatty acids requires a supply of reduced NADP. Therefore, the authors convinced that the elevation in an incorporation of $2\text{-}^{14}\text{C}$ -acetic acid into lipid fraction in the liver slices (Fig. 6) and the increased incorporation of ^{14}C -amino acids into lipid in the whole animals (Fig. 3) were caused by the increase in supply of NADPH through the G-6-P dehydrogenase system.

In the preceding paper (Ueno *et al.*, 1977a), the authors reported that the peptide caused *in vivo* a marked reduction in the contents of hepatic microsomal protein and glycogen. The present data also indicated a marked decline in the microsomal glycogen synthetase (Table 5). Therefore, the authors estimated the activity of drug-metabolizing enzymes, which are well known as a microsomal origin. The data obtained indicated that the microsomal mixed function oxygenases represented by aminopyrine demethylase and aniline hydroxylase also declined to 50-70 % of the control value (Table 1). From these results, the authors came to the conclusion that the peptide affects directly or indirectly the hepatic endoplasmic reticulum of mice and rats.

The mechanism of direct action of Cl-peptide to the hepatic E.R. system is unknown in the present time. The preceding tracer experiments (Ueno *et al.*, 1977a) have demonstrated that the s.c. administered peptide appeared in the blood stream, and over 20-30% of the total dose preferentially accumulated in the liver within 30 min after injection and remained in the liver for several hours thereafter. The relationship between this high affinity of Cl-peptide to the hepatic cells and the above-mentioned alteration of the E.R. system is remained to be disclosed.

SUMMARY

In vivo effects of chlorine-containing cyclic pentapeptide (chloropeptide) on the hepatic glycogen metabolism were investigated in various levels such as whole animals, liver slices and subcellular fractions. Administration of chloropeptide to mice and rats in a sublethal dose caused a marked decline in the hepatic glycogen synthetase activity with concomitant increase in NADP-dependent G-6-P dehydrogenase activity. No significant alterations in the activities of phosphorylase, G-6-P ase and F-1, 6-P phosphatase, aerobic and anaerobic glycolysis were demonstrated in the livers of mice and rats administered with chloropeptide. The increment in hepatic lipid synthesis following chloropeptide

treatment was caused by the accelerated supply of reduced NADP through the elevated G-6-P dehydrogenase in the hepatic cells. The activities of microsomal mixed function oxygenases represented by aminopyrine demethylase and aniline hydroxylase were reduced in the liver of rats following chloroptide treatment.

REFERENCES

- Barker, S.B., (1957) Preparation and colorimetric determination of lactic acid. In "Methods in Enzymology" (Colowick, S.P. and Kaplan, N.O. ed.), Vol. III, pp 447-454, Academic Press, New York.
- Hassid, W.Z. and Abraham, S., (1957). Chemical procedures for analysis of polysaccharides. In "Methods in Enzymology" (Colowick, S.P. and Kaplan, N.O. ed.), Vol. III, pp 34-50, Academic Press, New York.
- Imai, Y., Ito, A. and Sato, R., (1966). Evidence for biochemically different types of vesicles in the hepatic microsomal fraction. *J. Biochem.*, **60**, 417-429.
- Ishii, K. and Ueno, Y., (1973). Production of the hepatotoxic chlorine-containing peptide by *Penicillium islandicum* Sopp. *Appl. Microbiol.*, **26**, 359-363.
- Layne, E., (1957). Spectrophotometric and turbidimetric methods for measuring proteins. In "Methods in Enzymology" (Colowick, S.P. and Kaplan, N.O. ed.), Vol. III, pp 447-454.
- Leloir, L.F. and Cardini, C.E., (1957). Characterization of phosphorus compounds by acid lability. In "Methods in Enzymology" (Colowick, S.P. and Kaplan, N.O. ed.), Vol. III, pp 840-850, Academic Press, New York.
- Nash, T., (1953). The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. *Biochem. J.*, **55**, 416-421.
- Tatsuno, T., Tsukioka, M., Suzuki, Y. and Asami, Y., (1955). Recherche sur la substance toxique du riz janni. *Pharm. Bull. (Tokyo)*, **3**, 476-477.
- Ueno, Y., Ito, T., Hashimoto, N. and Ueno, I., (1977a). Acute toxicity of chloroptide, a hepatotoxic metabolite of *Penicillium islandicum* Sopp. *J. Toxicol. Sci.*, **2**, 349-362.
- Ueno, Y., Ito, T. and Ueno, I., (1977b). Hepatotoxicity and hepato-accumulation of cyclic peptide from *Penicillium islandicum* Sopp. *Proc. Ist. Intern. Congr. Toxicol.*, Toronto.
- Ueno, Y., Ito, T., Matsumoto, H., (1977c). Acute toxicity and metabolism of chloroptide, a hepatotoxic mycotoxin of *Penicillium islandicum* Sopp. *J. Jap. Assoc. Mycotoxicol.*, in press.
- Uraguchi, K., Tatsuno, T., Sakai, F., Tsukioka, M., Sakai, Y., Yonemitsu, O. and Ito, H., (1961). Isolation of two toxic agents, luteoskyrin and chlorine-containing peptide, from the metabolites of *Penicillium islandicum* Sopp, and some properties thereof. *Japan. J. Exp. Med.*, **31**, 19-46.
- Uraguchi, K., Saito, M., Noguchi, Y., Takahashi, K., Enomoto, M. and Tatsuno, T., (1972). Chronic toxicity and carcinogenicity in mice of the purified mycotoxins, luteoskyrin and cyclochlorotine. *Fd. Cosmet. Toxicol.*, **10**, 193-207.
- Yoshioka, H., Nakatsu, K., Sato, M. and Tatsuno, T., (1973). The molecular structure of cyclochlorotine, a toxic chlorine-containing cyclic pentapeptide. *Chemistry Letters*, 1319-1322.