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Differential Radioactivity Uptake from ^{14}C-Labeled D- and L-Leucine by the Pancreas of Animals pretreated with Pancreatitis-causing Agents[1)]

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It was demonstrated by *in vivo* and *in vitro* radioactivity determinations and autoradiographic studies that the pancreas of animals pretreated with carbon tetrachloride or ethionine exhibited a reduced radioactivity uptake upon ^{14}C-labeled L-leucine administration, whereas no significant decrease was observed in uptake of D-leucine. This uptake decrease from radioactive L-leucine in the pancreas of the treated animals is suggested to be due to inhibitions of protein synthesis and active membrane transport in the pancreas.

The present investigations suggest that comparative studies of amino acid uptake by the pancreas using ^{11}C-labeled D- and L-amino acids such as leucine might be more useful in the detection and assessment of pancreatitis by nuclear imaging of the pancreas than the use of L-amino acids alone or DL-amino acid racemates.

Keywords——amino acid uptake in pancreatitis models; uptake of amino acid in pancreatitis models; leucine uptake in the pancreas; pancreatitis model; CCl_4; ethionine; 3′-methyl-4-(dimethylamino)azobenzene; cycloheximide

Recently, clinico-chemical tests such as the determinations of amylase and peptidase activities have been developed for the *in vitro* diagnosis of pancreatitis, but the tests are insufficient for identification of the damaged region of the pancreas. Many investigations have been reported on the development of improved pancreas-imaging agents for the detection of the invaded region, even since the appearance of the widely used selenomethionine[^{75}Se]. Some of the present authors have found that among 18 radioactive amino acids, the D- or L-isomers of tryptophan, methionine, valine, leucine and phenylalanine could be the most useful as mother compounds in nuclear imaging of the pancreas with regard to *in vivo* uptake.[2)] Subsequently, the usefulness of radioactive amino acids as pancreas-imaging agents was demonstrated by the synthesis and animal studies of ^{11}C-labeled amino acids such as DL-valine, DL-leucine and DL-tryptophan by Hayes *et al.*[3)] and also by the ^{11}C-labeled methionine clinical studies of Syrota *et al.*[4)]

We herein report that the uptake of L-leucine decreased in the pancreas of animals after injection of pancreatitis-causing agents, whereas no significant decrease was observed for the D-form. The significance of this result is discussed in relation to pancreatitis diagnosis.

Materials and Methods

1. **Materials**——D-Leucine[1-^{14}C], L-leucine[U-^{14}C], and L-tryptophan[3-^{14}C] were products of ICN Pharmaceuticals, Inc., U.S.A., The Radiochemical Centre, U.K., and New England Nuclear, U.S.A., respectively. The radiochemical purities were checked by paper chromatography before use. Carbon tetrachloride, ethionine and other chemicals were of analytical grade. Wistar rats and ddY mice were purchased from Shizuoka Agricultural Cooperative Association for Laboratory Animals, Hamamatsu.

2. **Uptake Experiments *In Vivo***——2.1 *In Vivo* Uptake by CCl_4-damaged Rat Pancreas: Each group of 6 rats (4 weeks old and about 80—90 g body weight) received intraperitoneal injection of CCl_4 (0.2 or 0.04 ml/100 g body weight) dissolved in 0.3 ml of olive oil.[5)] Then, 4 h later the rats were subcutaneously or intravenously injected with radioactive D-leucine, L-leucine (3 μCi/200 nmol/0.2 ml saline/rat), or L-tryptophan

(0.6 μCi/200 nmol/0.2 ml saline/rat). Each control animal (6 rats/group) was first treated with 0.3 ml of olive oil and similarly injected with the D- or L-isomer of the labeled amino acid. Each group of rats was sacrificed by ether inhalation at an appropriate time after the amino acid administration, and organs to be tested were excised. After weighing of each organ, a small weighed portion (about 100 mg) of the organ was incubated in 1 ml of Protosol® (New England Nuclear) at 50—55°C overnight for dissolution. To the resultant clear tissue solutions, 10 ml of a scintillation cocktail [PPO (4 g) and POPOP (0.1 g) in 1000 ml of toluene] was added. In some cases a previously reported method[2b] of sample preparation was employed wherein the minced tissues were treated with trichloroacetic acid (TCA) followed by 2π gas flow counting of the TCA-soluble and -insoluble (crude protein) fractions.

2.2 *In Vivo* Uptake by the Pancreas of Mice fed a Choline-Deficient Diet Containing Ethionine: A group of 6 mice was starved for a day after receiving a choline-deficient diet for 2 d, then fed the same choline-deficient diet containing 0.5% ethionine for a day. After another day of the choline-deficient diet,[6] the mice were intravenously injected with ^{14}C-labeled D- or L-leucine (1 μCi/189 nmol/0.2 ml physiological saline/mouse). Six control mice were fed only conventional diet (Oriental Yeast Co., Ltd.). The mice were sacrificed 30 min after the injection and the organs were excised. Preparation of the samples for radioactivity determinations was carried out according to the procedure described above.

3. ***In Vitro* Uptake Experiments**——3.1 The pancreas from rats which had received the above described CCl_4 treatment was removed at 4 h after CCl_4 injection (0.2 ml/100 g body weight). About 150 mg of the sliced pancreas tissues was incubated for a definite time in 1 ml of Krebs–Ringer phosphate (KRP) buffer, pH 7.2, containing 0.1 μCi ^{14}C-labeled D- or L-leucine (1 μCi/μmol). The incubated tissues were washed 3 times with KRP buffer and treated according to the procedure described in section 2.1 for the radioactivity determination.

3.2 To determine the effect of cycloheximide on the pancreatic radioactivity uptake about 100—200

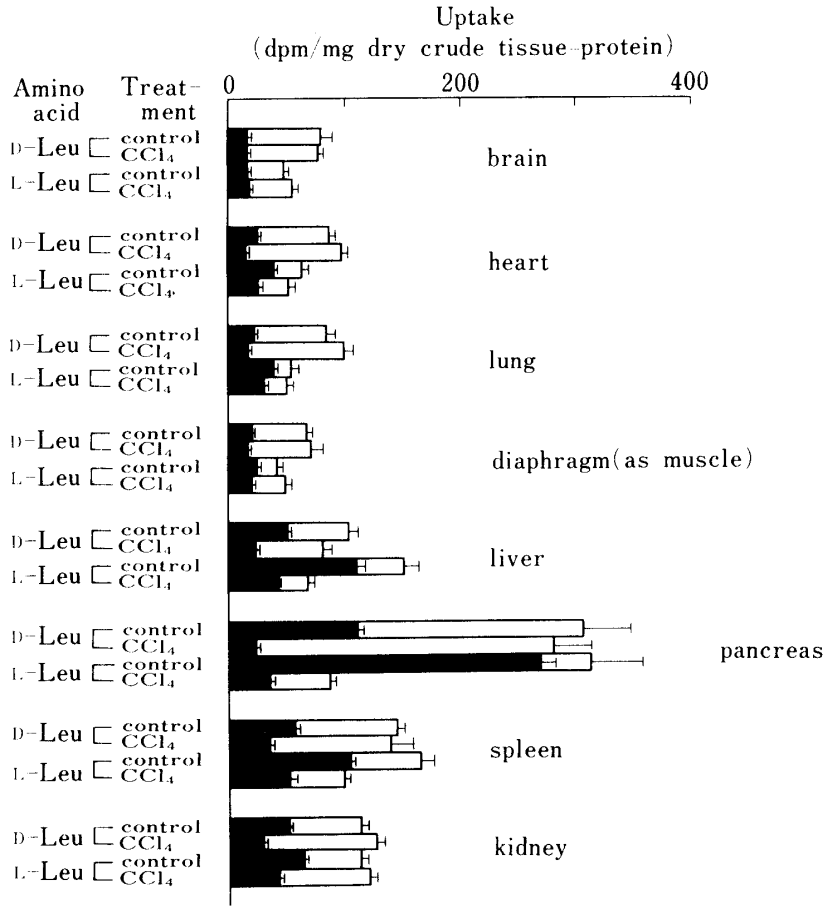

Fig. 1. Uptake of Radioactivity from ^{14}C-Labeled D- or L-Leucine by the Organs of CCl_4-injected Rats at 60 min after *s.c.* Injection of Amino Acid

A group of 6 rats was *i.p.* injected with CCl_4 (0.2 ml/100 g body weight) dissolved in 0.3 ml of olive oil, and 4 h later radioactive D- or L-leucine (3 μCi/200 nmol/0.2 ml saline/rat) was *s.c.* injected. Controls received 0.3 ml of olive oil. The rats were sarificed after 60 min.

■ : TCA-insoluble (crude protein) fraction, □ : TCA-soluble fraction, —| : standard deviation.

mg of the pancreas slices from normal mice (ICR swiss) was preincubated in triplicate at 37°C for 5 min with 0.5 ml of physiological saline (pH 7.2) containing cycloheximide (10^{-4} M). Then, the mixture was incubated for a given time with 0.5 ml of D- or L-leucine[^{14}C] (0.2 μCi/ml, 1 μCi/μmol) and treated as described above.

4. **Whole Body Autoradiographic Studies**——^{14}C-Labeled D- or L-leucine (20 μCi/400 nmol/0.4 ml physiological saline/rat) was subcutaneously injected into the backs of rats pretreated with carbon tetrachloride (0.2 ml/100 g body weight) as described in section 2.1. Sixty minutes later the rats were sacrificed by ether inhalation and whole body autoradiography was performed by the usual method using X-ray films.

5. **Histopathological Features of the Pancreas and Liver**——Histopathological features of the pancreas and liver from animals treated with pancreatitis-causing agents (CCl_4 or ethionine) at a given dose for the radioactivity uptake experiments were determined in the usual way by Dr. Shoji Hoshi, Director of the Clinical Pathology Department, Saiseikai Hospital, Shizuoka-shi.

Results

In Vivo Radioactivity Uptake of ^{14}C-Labeled D- or L-Leucine by Tissues of CCl_4-Treated Rats

Among the tissues tested the radioactivity uptake from L-leucine [U-^{14}C] was most markedly reduced in the pancreas of rats administered a CCl_4 dose of 0.2 ml/100 g body weight, while the uptake from D-leucine [1-^{14}C] was only slightly reduced (Fig. 1). A slight reduction was also observed for the liver and spleen, especially in the case of the L-isomer. Interestingly,

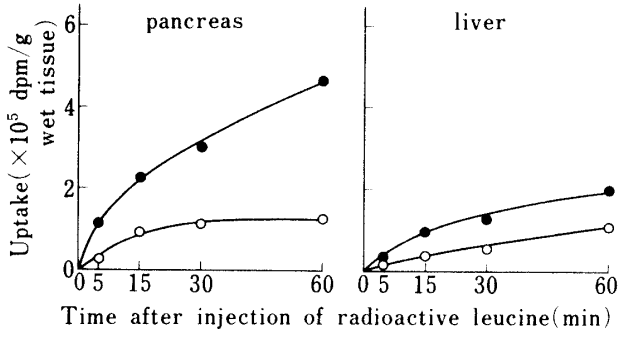

Fig. 2. Time Course of Radioactivity Uptake from ^{14}C-Labeled L-Leucine by the Pancreas and Liver of Rats Pretreated with CCl_4

Experimental procedure was as described in the legend to Fig. 1.
●: control, ○: CCl_4-treated.

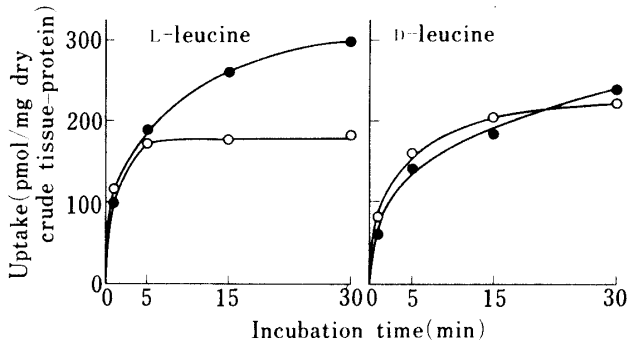

Fig. 3. *In Vitro* Uptake of Radioactivity from D- or L-Leucine by the Sliced Pancreas of Rats Treated with CCl_4

Approximately 150 mg of pancreas slices from rats which had received CCl_4 (0.2 ml/100 g body weight) at 4 h prior to killing was incubated with 1 ml of Krebs-Ringer phosphate buffer (pH 7.2) containing 0.1 μCi D- or L-leucine (1 μCi/μmol) at 37°C.
●: control, ○: CCl_4-treated.

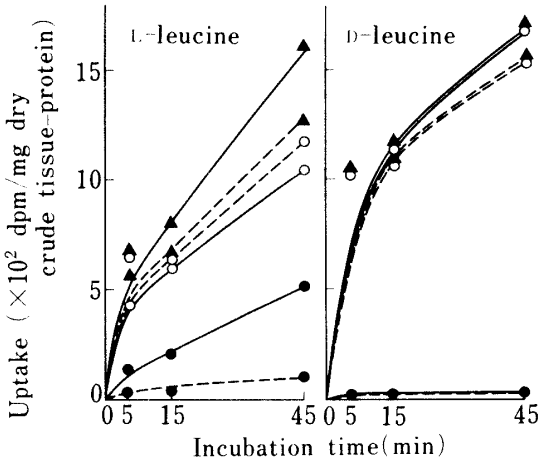

Fig. 4. Effects of Cycloheximide on Radioactivity Uptake from ^{14}C-Labeled D- or L-Leucine by Minced Mouse Pancreas

About 100—200 mg of pancreas slices was preincubated at 37°C for 5 min with 0.5 ml of physiological saline (pH 7.2) containing cycloheximide (10^{-4} M), and the mixture was incubated with 0.5 ml of D- or L-leucine[^{14}C] (0.2 μCi/ml, 1 μCi/μmol). The TCA-insouble fractions were burned in a sample oxidizer (Packard). The radioactivity of TCA-soluble and -insoluble fractions was determined with a scintillation counter (Packard).

——: control (no cycloheximide), ·····: cycloheximide,
○: TCA-soluble fraction, ●: TCA-insoluble fraction,
△: TCA-(soluble+insoluble) fraction.

no such reduction in either D- or L-leucine was seen in other tissues tested. Furthermore, it is noteworthy that in the CCl_4-damaged rat pancreas, the radioactivity uptake from L-leucine [U-^{14}C] was markedly reduced in the protein fraction, but not in the TCA-soluble fraction. On the other hand, reduced uptake from D-leucine [1-^{14}C] was seen only in the protein fraction and uptake in the TCA-soluble fraction appeared to be slightly increased. Figure 2, in which pancreas and liver radioactivity uptake is indicated as a function of time after injection of ^{14}C-labeled L-leucine, shows essentially the same results as obtained in the above experiment.

In another experiment in which ^{14}C-labeled D- or L-leucine was intravenously injected 30 min prior to killing, the same results for the pancreas and liver were obtained as in the above experiment in which the radioactive leucine was subcutaneously injected 60 min prior to sacrifice.

No reduction in pancreas radioactivity uptake from L-leucine [U-^{14}C] was observed when the rats received a CCl_4 dose of 0.04 ml/100 g body weight.

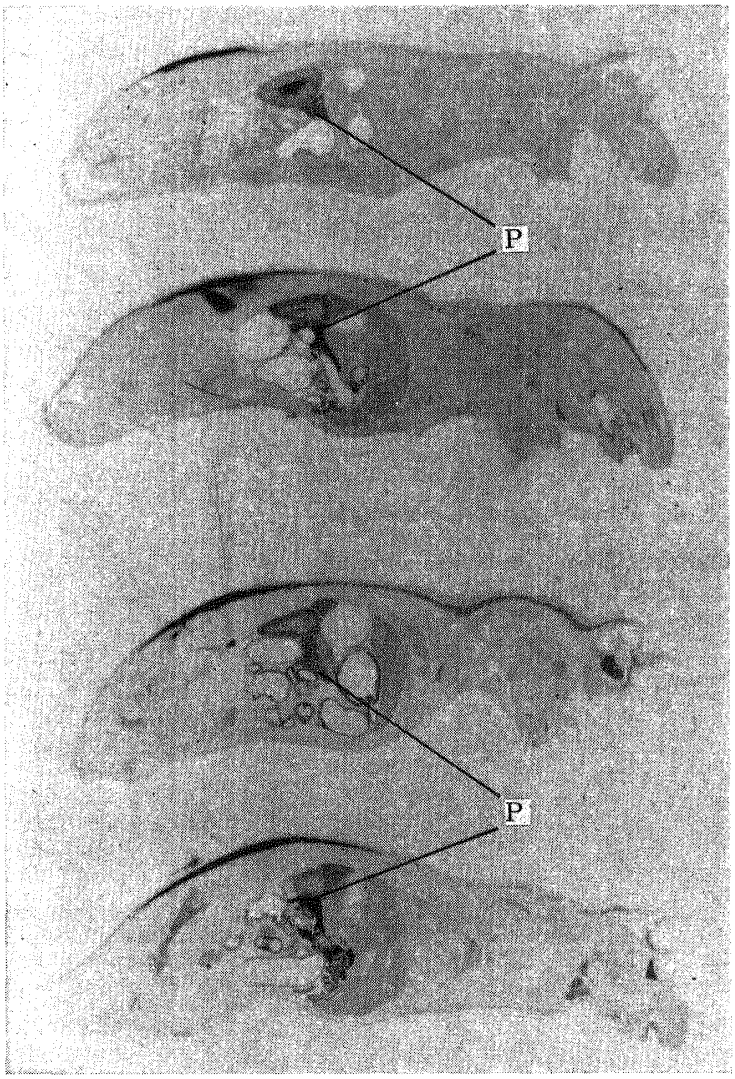

Fig. 5. Autoradiographic Illustration of the Effect of Carbon Tetrachloride on the Tissue Distribution of Radioactivity from D- and L-Leucine[^{14}C]

Autoradiographs I and II show the distributions of radioactivity from D-leucine-[1-^{14}C] in CCl_4-treated and control (olive oil treatment) rats, respectively, and III and IV show the dispositions from L-leucine[U-^{14}C] in CCl_4-treated and control rats, respectively.
P: pancreas.

The uptake of L-tryptophan [3-^{14}C] also fell to about one-third in the pancreas of rats pretreated with CCl_4.

In Vitro Radioactivity Uptake by the Sliced Pancreas from Rats pretreated with CCl_4

Figure 3 depicts the radioactivity uptake of ^{14}C-labeled D- and L-leucine as a function of incubation time, showing reduced uptake of L-leucine [U-^{14}C] in pancreas slices from CCl_4-treated rats with practically no change for the D-isomer.

As expected from the low uptake in the TCA-insoluble fraction indicated in Fig. 1, a reduction in L-leucine [U-^{14}C] uptake by minced mouse pancreas in the presence of cycloheximide, an inhibitor of protein synthesis in mammalian cells,[7] was mainly found in the TCA-insoluble (crude protein) fraction (Fig. 4), in accord with the results observed in the *in vivo* experiment (Fig. 1) using CCl_4-treated rats.

Whole-Body Autoradiographic Studies

Autoradiographic studies of rats which had received CCl_4 at a dose of 0.2 ml/100 g body weight clearly showed reduced pancreatic uptake of L-leucine [U-^{14}C] (Fig. 5) as observed in the above reported *in vivo* uptake.

In Vivo Uptake of Radioactive Leucine by the Pancreas of Ethionine-administered Mice on a Choline-Deficient Diet

A reduced uptake of L-leucine [U-^{14}C] was observed in the pancreas from mice which had been administered with ethionine, an alleged pancreatitis-causing agent (Fig. 6). No such reduction of radioactivity uptake was found in the case of D-leucine [1-^{14}C].

Histopathological Features of the Pancreas and Liver from Animals treated with Pancreatitis-causing Agents

As shown in Table I, minor histopathological changes could be seen in the liver of CCl_4-administered rats compared with those of untreated animals, but no differences were observed

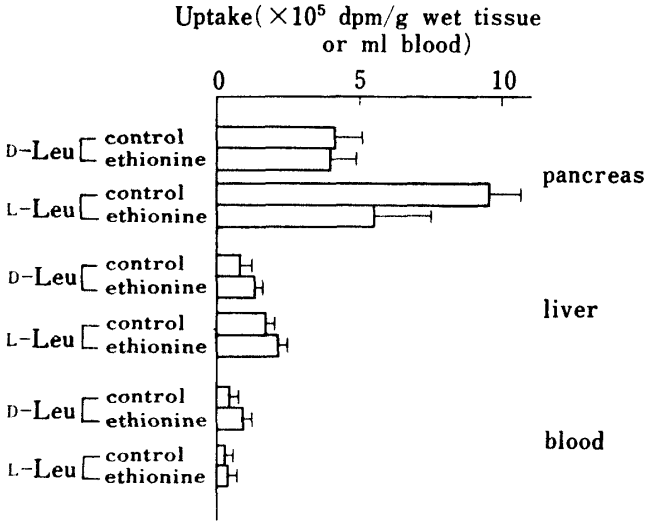

Fig. 6. Radioactivity Uptake from ^{14}C-labeled D- or L-Leucine by the Tissues of Mice treated with Ethionine and fed on a Choline-Deficient Diet

A group of 6 mice which had been starved for a day after being fed a choline-deficient diet for 2 d was fed with the same choline-deficient diet containing 0.5% ethionine for a day. Then, after further feeding of the deficient diet for a day, the mice were *i.v.* injected with 1 μCi ^{14}C-labeled D- or L-leucine (1 μCi/189 nmol/mouse) dissolved in 0.2 ml of saline. The mice were sacrificed after 30 min.

⊣: standard deviation.

Table I. Histopathological Features of the Liver and Pancreas of Rats 4 h after *i.p.* Injection of Carbon Tetrachloride dissolved in 0.3 ml of Olive Oil

Tissue	Histopathological feature	Control	ml of CCl_4/100 g body weight				
			0.2			0.04	
		Animal No.					
		1	2	3	4	5	6
Liver	Cellular infiltration of Glisson's capsules	−	−	−	−	−	−
	Swelling of cells	−	−	−	−	−	−
	Atrophy of cells	−	−	−	−	−	−
	Degeneration or necrosis of cells	−	±	−	−	−	±
	Appearance of fat droplets	−	−	±	±	+	±
	Cellular infiltration in the anatomic lobules	−	±	−	−	−	−
	Hyperemia or bleeding	−	−	−	−	−	−
Pancreas	Degeneration or disintegration of Langerhans' cells	−	−	−	−	−	−
	Atrophy of exocrine gland	−	−	−	−	−	−
	Disintegration of exocrine gland	−	−	−	−	−	−
	Cellular infiltration	−	−	−	−	−	−
	Hyperplasis of the stroma	−	−	−	−	−	−

The symbols −, ± and +: denote nil, negligible, and weak changes, respectively.

Table II. Histopathological Features of the Liver and Pancreas of Mice which had received Ethionine on a Choline-Deficient Diet

Tissue	Histopathological feature	Animal No.		
		1	2	3
Liver	Fatty degeneration	++	+++	+++
	Necrosis	±	±	+
	Cellular infiltration	+	±	±
Pancreas	Vacuolar degeneration	+	+	++
	Necrosis	−	−	−
	Cellular infiltration	−	−	−
	Degeneration of the Langerhans' cells	−	−	−

The symbols −, ±, +, ++ and +++: signify nil and, in ascending order, increasingly positive degrees of histopathological changes.

in the pancreas. In ethionine-treated mice, severe fatty degeneration in the liver and a weak or moderate degree of vacuolar degeneration in the pancreas were seen (Table II).

Discussion

It is well known that L-amino acids are avidly utilized by the pancreas.[2,9] D-Amino acids are primarily taken up into the TCA-soluble amino acid pool, not into the protein fraction.[2,9d] Carbon tetrachloride and ethionine are pancreatitis-causing agents[6,10] as well as inhibitors of protein synthesis.[11] Recently, a reduced uptake of L-leucine [U-^{14}C] was observed in the pancreas of rats fed for 150 days on a diet containing 0.06% 3′-methyl-4-(dimethylamino)azobenzene (3′-Me–DAB), but no such effect was seen for D-leucine [1-^{14}C].[8]

The reduced radioactivity uptake from L-leucine [U-^{14}C] by the pancreas of animals treated with the pancreatitis-causing agents CCl_4 or ethionine is best explained in terms of inhibition of protein synthesis as indicated by the lower amount of radioactivity found in the TCA-insoluble fraction from the pancreases of CCl_4-treated (Fig. 1) and 3′-Me–DAB-treated rats.[8] In agreement with this explanation is the finding that the radioactivity uptake from D-leucine-[1-^{14}C] was not significantly influenced by CCl_4 (Fig. 1) or 3′-Me–DAB.[8] This explanation is also supported by an *in vitro* experiment in which cycloheximide was used to depress protein

synthesis in the normal mouse pancreas (Fig. 4). However, a large difference in L-leucine uptake, as was found in the pancreases of the CCl_4-treated and -untreated animals (Fig. 1), was not observed in this experiment, because a large proportion of radioactivity from ^{14}C-labeled L-leucine was detected in the TCA-soluble fraction (Fig. 4). This means that cycloheximide affects only the protein synthesis but not the membrane transport. Therefore, it is suggested that the pancreatitis-causing agents used in this experiment acted not only on protein synthesis, but also on membrane transport of L-leucine. In fact, Recknagel reported that CCl_4 disrupted the membranous components of the cytoplasm.[11a] Furthermore, there is a possibility that the energy-requiring membrane transport of L-leucine is also affected by these agents, because Dianzani has reported that CCl_4 inhibited oxidative phosphorylation in the mitochondria of liver cells.[12]

It is very interesting that in animals pretreated with pancreatitis-causing agents the reduction of radioactive L-leucine uptake by the pancreas occurred prior to the appearance of severe histopathological changes in the pancreatic tissues (Tables I and II).

Clinical investigations by Syrota *et al.* on pancreatitis demonstrated that uptake of ^{11}C-labeled methionine synthesized from L-homocysteine and $^{11}CH_3I$ was decreased in pancreatitis.[4] Hübner *et al.* reported that inadequate pancreatic function was indicated by poor uptake of ^{11}C-labeled tryptophan.[3c] It is expected from the present studies that uptake of radioactivity from D-amino acids would be impaired only in advanced stages of pancreatitis. Accordingly, we conclude on the basis of the present investigation that comparative studies of amino acid uptake by the pancreas using ^{11}C- or γ-emitter-labeled D- and L-amino acids such as leucine, tryptophan, methionine, valine, and phenylalanine already reported[2] might be more useful in the detection and assessment of pancreatitis by nuclear imagning of the pancreas than would the use of L-amino acids alone or DL-amino acid racemates. Also, it is suggested that the comparative uptake determination of ^{14}C-labeled D- and L-amino acids could be extended as an auxiliary tool in experimental animals to evaluate the effect of pancreatitis-curing drugs. Fu thermore, the results of the present studies might be valuable in estimating the inhibitory effect of protein synthesis in the pancreas and consequently in predicting the toxicity to the pancreas of drugs and other chemical substances in animals and ultimately in humans.

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References and Notes

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