STUDIES ON CAERULEIN (FI6934). ABSORPTION, DISTRIBUTION, METABOLISM AND EXCRETION OF CAERULEIN

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Abstract—35S-Caerulein (35S-F16934) was administered intramuscularly into rats (280 μg/kg), rabbits (380 μg/kg) and mice (3.3 mg/kg). Blood level of radioactivity in rats and rabbits reached the maximum at 5 and 15 min after administration, respectively, and then decreased rapidly. In both rats and rabbits, the radioactivities were excreted mainly into the urine. The physiological activity of FI6934 was detected in the blood of rats and rabbits collected within 15 and 30 min after injection respectively, and in the bile of rats collected within the first 2 hr. In rats, the radioactivities were densely distributed in kidney, liver, pancreas, and intestine. Four metabolites of 35S-FI6934 were isolated by paper chromatography from rat urine (i.m., 500 μg/kg). The main metabolites, F-I and F-II, were negative to ninyhydrin and to the Ehrlich reagent and carried no physiological activity. 35S-Chym-I, which was prepared by digesting 35S-FI6934 with chymotrypsin, was injected to rats (i.m. 16 or 8 mg/kg). The metabolite, C-I, isolated from the urine was considered to have a very similar structure to F-I from the results of paper chromatography and paper electrophoresis. By amino acid analysis, the structure of C-I was estimated to be as follows:

Pyr—Gln (or Glu)—Asp—Tyr—Thr—Gly

*SO₃H

F16934 (Caerulein*) is a decapeptide discovered by Erspamer et al. (1) and Anastasi et al. (2) in the skin of Australian frog, Hyla caerulea, and known to have physiological activities similar to those of gastrin and cholecystokin-in-pancreozymin. That is, the peptide accelerates the gastric secretion (3–5), the pancreatic secretion (6–9), and induces the contraction of intestinal and gallbladder smooth muscle (9–11). The sequence of C-terminal pentapeptide of FI6934 is the same as that of human gastrin II and cholecystokin-in-pancreozymin (12). The peptide also has the tyrosil sulfate moiety as well as human gastrin II (12).

Structure of F16934

**Pyr—Gln—Asp—Tyr—Thr—Gly—Try—Met—Asp—Phe—NH₂

*SO₃H

* ceruletide—International Non Proprietary Name (INN)
** In this paper the following abbreviations were used: Pyr; Pyroglutamic acid, Gln; Glutamin, Glu; Glutamic Acid, Asp; Asparatic acid, Tyr; Tyrosine, Tyr-SO₃H; Tyrosil sulfate, Thr; Threonine, Gly; Glycine, Try; Tryptophan, Met; Methionine, Phe; Phenylalanine, Phe-NH₂; Phenylalanine-NH₂, Ser; Serine.
The fate of F16934 administered into the animal body offers very interesting problems in relation to the physiological activities of the peptide. In general, it is very difficult, however, to trace the fate of peptide in the body, since it is hydrolysed easily by various enzymes and there are also present many other peptides in the biological samples. In particular, the effective concentration of F16934 required to demonstrate its physiological activities is so low that it is almost impossible to investigate the metabolism of the peptide in vivo at a concentration within the effective range.

In this report, we describe the study on the absorption, distribution, metabolism and excretion of F16934 when administered i.m. to animals at concentrations more than 100 times as high as the physiologically effective level, by using $^{35}$S-labeled F16934 ($^{35}$S-F16934) as a tracer, which was obtained by labeling its tyrosylsulfate moiety with radioactive sulfur ($^{35}$S), together with a physiological assay based on the activity of the peptide to induce the contraction of guinea pig gallbladder (11).

MATERIALS AND METHODS

Reagent: We used F16934 synthesized by Farmitalia. According to the amino acid analysis (12) (13), the F16934 contained no other amino acids than its constituents (see Table 5.)

$^{35}$S-F16934 that was used in this study was synthesized by the method described in a previous report (14). The radiochemical purity of the peptide was determined to be more than 99% by paper chromatography (14). The stock solution of $^{35}$S-F16934 was stored at $-30^\circ$C in a deep freezer.

The $^{35}$S-Chym-I (12) used in this experiment was obtained by digesting $^{35}$S-F16934 with chymotrypsin by the method described below. Five mg of F16934 and 0.97 mg of chymotrypsin were dissolved in 1 ml of 0.5 M ammonium acetate solution (pH 8.2), to which 40 $\mu$l of $^{35}$S-F16934 (0.1 mCi/ml) was added and allowed to react for 4 hr at 37°C. After the reaction was stopped by putting the reaction mixture in a hot bath, the mixture was centrifuged. $^{35}$S-Chym-I in the supernatant was separated and purified by paper chromatography. Its amino acid composition was determined to be (Pyr—Glu, Asp, Tyr, Gly, Trp) by amino acid analysis (see Table 5). This peptide did not carry the physiological activity to induce the contraction of the guinea pig gallbladder.

Experimental animals: Sprague-Dawley strain rats (male, weighing an average of 250 g each) and Japanese White rabbits (male, weighing an average of 3 kg each) were used.

Administration of $^{35}$S-F16934: In cases of the studies of absorption, distribution and excretion of F16934, the stock solution of $^{35}$S-F16934 was diluted before use with physiological saline and injected intramuscularly into a hind leg of each animal in a dose of 2-1 $\mu$Ci per rat (280 $\mu$g/kg), and 10 $\mu$Ci per rabbit (380 $\mu$Ci/kg). For the study of metabolism, 2.5-3.6 $\mu$Ci of $^{35}$S-F16934 diluted with cold F16934 was injected intramuscularly into the rat (0.5 or 5 mg/kg).

Determination of radioactivity in the blood of rats: 100 $\mu$l of the blood was collected from the tail vein of each animal into a glass vial 5, 10, 15, 30 and 60 min, and 2, 3, 4, 5, 6
and 24 hr after administration of $^{35}$S-F16934. To the sample were added 100 μl of perchloric acid and 200 μl of hydrogen peroxide, and heated in a water bath at 70°C until the solution decolored. Twenty ml of scintillator B (a mixture of 1 l of toluene containing 6 g of PPO and 500 ml of ethylene glycol monomethyl ether) was then added, and the radioactivity was measured with a liquid scintillation counter (Packard Model 3380).

**Determination of radioactivity excreted in the urine and feces of rats:** Each of the three rats administered $^{35}$S-F16934 was housed in a metabolic cage. The urine and feces were sampled at 8 and 24 hr. For determining the radioactivity in the urine, 100 μl of the sample was mixed with 10 ml of scintillator F-II (a mixture of 730 ml of dioxane containing 100 g of naphthalene, 5 g of PPO and 0.3 g of dimethyl POPOP, and 135 ml of toluene and 35 ml of methanol.) Feces were homogenized with 5-fold of distilled water (w/w) in a VirTis homogenizer at 45,000 rpm for 5 min. A hundred mg of the homogenate, weighed into a glass vial, was heated at 70°C with 100 μl of perchloric acid and 200 μl of hydrogen peroxide until the solution is decolored, mixed with scintillator B, and used for determination of the radioactivity. Twenty-four hr after administration of $^{35}$S-F16934, the rats were sacrificed by bleeding under ether anesthesia, and the following organs were dissected: brain, heart, lungs, liver, kidneys, adrenals, stomach and its contents, intestine and its content, spleen, pancreas, muscles, fat bodies, testis and blood. These organs and carcass were homogenized with the same weight or two-fold the weight of distilled water in a VirTis homogenizer at 45,000 rpm for 10 min. Two hundred mg of the homogenate, weighed into a glass vial, was decomposed by adding 2 ml of Soluene 100 (Packard) and heating at 40°C for several hr, mixed with 10 ml of scintillator A (prepared by dissolving 4 g of PPO and 0.25 g of dimethyl POPOP in 1 l of toluene), and used for measurement of the radioactivity. The content of stomach and intestine was homogenized with 5 and 10 ml of distilled water, respectively, in a VirTis homogenizer. The homogenate (100 mg), weighed into a glass vial, was decomposed by heating with 100 μl of perchloric acid and 200 μl of hydrogen peroxide at 70°C, and used for determination of the radioactivity after mixing with 15 ml of scintillator B.

**Determination of the radioactivity in the bile of rats:** Each of three rats whose bile ducts were connected with cannulae, was fixed in a restraint cage to collect the bile in each period of 0-0.5, 0.5-1, 1-2, 2-3, 3-4, 4-5, 5-20 and 20-24 hr after administration of the drug. Fifty μl each of the bile so collected was mixed with 10 ml of scintillator F-II, and used for determination of the radioactivity.

**Distribution of radioactivity in rats:** Five rats to which $^{35}$S-F16934 had been given were sacrificed by bleeding under ether anesthesia, 15, 30, 60, 90 and 120 min after administration, respectively, and the following organs were removed: brain, liver, kidneys, pancreas, stomach, duodenum, jejunum, ileum, cecum and colon. Each of the samples was treated as mentioned above and used for determination of the radioactivity. The same experiment was repeated 3 times, and an average value of measurements in each three rats sacrificed at a corresponding time was obtained.

**Determination of radioactivity in the blood of rabbits:** $^{35}$S-F16934 was given to 2 rabbits,
and 100 μl each of the blood was collected from the ear vein of each animal after 5, 10, 15, 30 and 60 min, and 2, 3, 4, 5 and 24 hr. The radioactivity was measured in the same manner as in the case of rats.

**Determination of radioactivity excreted from rabbits:** Two rabbits were given 35S-F16934 and housed in metabolic cages to collect the urine and feces after 8 and 24 hr. The radioactivity was measured in the same manner as in the case of rats.

**Determination of radioactivity in the bile and the pancreatic fluid of rabbits:** Cannulae were inserted into both bile and pancreatic ducts of two rabbits under anesthesia with pentobarbital, and the animals were allowed to stand for about 1 hr to collect bile and pancreatic fluid at intervals of 15 min and 30 min, respectively. Then 35S-F16934 was administered, and bile was collected from the animals in each period of 0–15, 15–30, 30–45, 45–60, 60–75, 75–90, 90–105 and 105–120 min, and 2–3, 3–4, 4–5 and 5–6 hr after administration. Pancreatic fluid was collected in each period of 0–1, 1–3, 3–4, 4–5 and 5–6 hr. For measuring the radioactivity, 10 ml of scintillator F-II was added to 100 μl of the bile, and 10 ml of scintillator A was added to 50 or 100 μl of pancreatic fluid mixed with 2 ml of Soluene 100.

**Determination of bilirubin index in rabbits:** According to Meulengracht's method, the absorbance of the bile diluted with a 0.9% NaCl solution to 1:20 was measured at 420 nm against a 0.01% potassium bichromate solution taken as the standard (15).

**Determination of protein concentration in the pancreatic fluid of rabbits:** The protein concentration was determined by the biuret reaction. (16)

**In vitro reaction of F16934 with the blood:** 10 μl of F16934 (0.12 mg/ml) was added to 600 μl of the heparin-treated rat blood and allowed to react at 37°C for a definite period of time. After stopping the reaction by heating the mixture at 100°C for 1 min, 3 ml of Tyrode solution was added to this mixture. The supernatant fluid obtained by centrifuging the reaction mixture was diluted 100-fold and subjected to bioassay to determine the F16934 concentration.

**In vitro reaction of F16934 with tissue homogenates:** The kidneys and liver, isolated from decapitated rats were homogenized with 3 volumes of physiological saline by a glass homogenizer with a teflon pestle. The kidneys and liver homogenate thus obtained was diluted with 20-fold and 10-fold water, respectively. The respective solution was allowed to react with 35S-F16934 at 37°C for a definite period of time and then cooled with ice to stop the reaction and centrifuged. An aliquot of the supernatant fluid thus obtained was taken to assay the F16934 concentration physiologically.

**Analysis of metabolites in the rat urine:** After injecting F16934 intramuscularly into rats, the urine were taken within 5 hr. The collected urine was applied directly on papers for paper chromatography and paper electrophoresis.

35S-Chym-I was also injected i.m. into rats and the urine collected was analyzed by paper chromatography and paper electrophoresis. The structure of the metabolites isolated by paper chromatography and paper electrophoresis was determined by amino acid analysis.

**Paper chromatography:** Filter paper No. 51A (Toyo Roshi) was used. Descending
chromatography were carried out by using 80% ethanol (the solvent 80%) or a mixture of n-butanol : pyridine : water (1 : 1 : 1, v/v) (the solvent 1 : 1 : 1) as a developing solvent. As for the staining reagents, we used the Ehrlich reagent for tryptophan and ninhydrine for free α- and ε-amino groups. The extent of migration of respective metabolite was expressed as the ratio of its migrated distance to that of 35S-F16934 (RF1).

**Paper electrophoresis:** On the Toyo Roshi filter paper No. 51A, a sample solution was spotted and the electrophoresis was carried out for one or two hr, in a solvent of 80% formic acid : acetic acid : water (5 : 15 : 80, v/v) at pH 1.7 and 1500 V(=35 V/cm). The extent of migration of respective metabolite was expressed as the ratio of its migrated distances to that of cysteine-SO₃H at pH 1.7 (1.7 Ecys).

**Biological assay of FI6934 in the blood, urine and bile of rats:** The biological assay was carried out by measuring the contraction of guinea pig gallbladders according to the method of Nakamura et al. (11).

The urine and blood of rats contained FI6934-like substances which induce the contraction of gallbladders. Therefore, these samples were diluted before use for bioassay to the extent that the effect of such substances on the contraction of gallbladder could be disregarded (1 : 50 for the urine and 1 : 200 for the blood.) The urine was diluted with Tyrode's solution. In the case of the blood, 200 µl of the blood was hemolyzed by adding the same volume of distilled water, mixed with 40 ml of Tyrode's solution and centrifuged to use the supernatant for measuring the gallbladder contraction.

The bile of rats contained no biological component which has an FI6934-like physiological activity.

**Biological assay of FI6934 in the blood, bile and pancreatic fluid of rabbits:** Samples collected from the 35S-FI6934 administered rabbits were used for measurement in the same manner as the bioassay in rats. To eliminate the effect of the FI6934-like biological components in these samples, the blood, bile and pancreatic fluid was diluted to 1 : 250, 1 : 50 and 1 : 100 or more, respectively, before use.

**RESULTS**

**Absorption, distribution and excretion in rats**

**Radioactivity in the blood:** Fig. 1 shows the time-course of the radioactivity change in the whole blood from the 35S-FI6934 administered rats. The amount of the whole blood was calculated to be 1/13 of the body weight of rat (17). The radioactivity decreased relatively rapidly during the first 4 hr, and then increased slightly at 6 hr after administration. The radioactivity lowered to less than 2 % of the maximum level in the blood at 24 hr after administration. In this figure, the changes in the concentration of FI6934 in the blood determined by the bioassay are also shown. The physiological activity of FI6934 administered to rats decreased so rapidly that the concentration of FI6934 in the blood collected 30 min after administration could not be detected by bioassay.

**Excretion and retention of radioactivity:** Table 1 shows the excretion of radioactivity in the urine and feces up to 24 hr after injection of 35S-FI6934 and the radioactivity retained
FIG. 1. Changes in the blood level of Fl6934 administered to rats (280 μg/kg)
- - concentration of Fl6934 determined by measuring radioactivity
  (each point represents the mean of 3 animals)
- - concentration of Fl6934 determined by bioassay
  (each point represents the mean of 2 animals)

| Period (hr) | Urine          | Feces         | Carcass       |
|------------|----------------|---------------|---------------|
| 0 – 8      | 80.19 ± 4.96*  | 0.80 ± 0.13   |               |
| 0 – 24     | 88.00 ± 1.55   | 4.54 ± 1.22   | 2.48 ± 0.75   |

*Percent of dose recovered. Mean ± standard deviation of 3 experiments

Radioactivity in the bile: Table 2 demonstrates the time course of the bile excretion of the radioactivity up to 24 hr after injection of $^{35}$S-Fl6934. The radioactivity excreted into the bile up to 24 hr after injection was 13.00% of the administered dose. In the bile collected in the first 2 hr, the physiological activity of Fl6934 was present, and the concentration of Fl6934 determined by bioassay were in good agreement with that determined by measuring radioactivity. In the 2 to 3 hr bile, however, the physiological activity of...
TABLE 2. Biliary excretion of radioactivity after injection of $^{35}$S-FI6934 to rats (280 μg/kg)

| Period (hr) | Excretion (mean and standard deviation of 3 animals) |
|-------------|---------------------------------------------------|
|             | Percent of dose administered (%)                  |
|             | Cumulative excretion (%)                          |
| 0 - 1       | $8.76 \pm 1.06$                                   | $8.76 \pm 1.06$ |
| 1 - 3       | $3.32 \pm 0.18$                                   | $12.08 \pm 0.92$ |
| 3 - 4       | $0.29 \pm 0.07$                                   | $12.37 \pm 0.98$ |
| 4 - 5       | $0.14 \pm 0.05$                                   | $12.51 \pm 1.03$ |
| 5 - 20      | $0.45 \pm 0.19$                                   | $12.96 \pm 1.16$ |
| 20 - 24     | $0.04 \pm 0.02$                                   | $13.00 \pm 1.15$ |

FI6934 could not be detected.

Distribution of radioactivity in the tissues: Fig. 2 demonstrates the changes in the radioactivity with time in the rat tissues up to 2 hr after administration of $^{35}$S-FI6934. The radioactivity in dpm per g of wet weight of the tissues was plotted against the time.

![Fig. 2. Distribution of radioactivity in tissues of rats after injection of $^{35}$S-FI6934 (280 μg/kg)
(each point represents the mean of 3 animals)

- ■, brain;
- ○, liver;
- ◇, kidneys;
- ▲, pancreas;
- ●, stomach;
- ●, duodenum;
- △, jejunum;
- ×, ileum;
- ▲, cecum;
- □, colon.](image-url)
highest radioactivity was found in the kidneys. High radioactivity was also detected in the liver, pancreas, duodenum, jejunum and ileum. In such organs as the brain, cecum and colon, the radioactivity changed with time in parallel with the radioactivity change in the blood, showing a relatively rapid decrease in a period from 15 min to 2 hr after administration. By contrast, the radioactivity decreased slowly in the kidneys, liver, pancreas, stomach, duodenum and jejunum. Especially, the radioactivity in the kidneys, and duodenum increased slightly in the period from 15 to 30 min after administration. In the ileum, the radioactivity increased gradually until 2 hr after administration.

**Absorption and excretion in rabbits**

**Radioactivity in the blood:** The changes in the radioactivity and physiological activity in the blood from the $^{35}$S-F16934 administered rabbits were determined. The concentration of F16934 in the blood, calculated from the results obtained in these two different experiments, were plotted against the time after administration of $^{35}$S-F16934 (Fig. 3). The time-course of the change in the radioactivity showed a maximum value at 15 min after administration, followed by a gradual decrease. The physiological activity of F16934 could be measured in the blood until 30 min after administration. Although the concentration of F16934 assayed biologically was nearly the same as that obtained by the radioactivity determination until 15 min after administration, the former was about 1/2 of the latter in the blood collected at 30 min.

**Radioactivity in the bile, the bile secretion and bilirubin index:** Fig. 4 illustrates the time-course of the excretion of radioactivity in the bile from the $^{35}$S-F16934 administered rabbits. The peak of radioactivity was seen in the 30 to 45 min bile. The total radioactivity excreted in the bile up to 6 hr after administration was 1.61 % of the dose administered. In this figure, the volume of the bile secreted in the unit period and the bilirubin index of each sample were also plotted against the time. The bile secretion rate after administration of $^{35}$S-F16934 was almost unchanged for the first 45 min, but it decreased significantly for the period from 45 min to 6 hr. The bilirubin index decreased temporarily for the first 1 hr, and then increased gradually. The bilirubin index in the 5 to 6 hr bile showed a level higher than that in the sample collected before administration of $^{35}$S-F16934. The physiological activity of F16934 could not be detected in the bile thus collected.

**Radioactivity and protein in the pancreatic fluid, and the pancreatic fluid secretion:**
FIG. 4. Excretion of radioactivity in bile, and changes in the bile secretion rate and bilirubin index after injection of $^{35}$S-F16934 to rabbits (380 μg/kg, mean of 2 animals)

FIG. 5. Secretion of radioactivity in pancreatic fluid, and changes in the secretion rate of pancreatic fluid and protein after injection of $^{35}$S-F16934 to rabbits (380 μg/kg, mean of 2 animals)

dependent changes of the radioactivity and the amounts of protein in the pancreatic fluid, and the pancreatic fluid secretion rate were examined in the $^{35}$S-F16934 injected rabbits (Fig. 5). The radioactivity increased slowly after administration of the peptide showing a peak in 3 to 4 hr. The total radioactivity secreted in 6 hr after administration was only 0.035 % of the administered dose. The amount of protein in the pancreatic fluid increased for the first hr, and then decreased in 1 to 3 hr showing a level lower than that before administration of $^{35}$S-F16934. In the 3 to 5 hr pancreatic fluid, the amount of protein increased to reach almost the same level as the original. The pancreatic fluid secretion rate decreased significantly until 3 hr after administration, and increased again in 3 to 6 hr. As in the case of the bile, the physiological activity of F16934 could not be detected in the pancreatic fluid collected from the $^{35}$S-F16934 injected rabbits.

Radioactivity excreted in the urine and feces: The excretion of the radioactivity in the
TABLE 3. Excretion of radioactivity from rabbits after injection of \( ^{35} \text{S-FI6934} \) (mean of 2 animals)

| Period (hr) | Excretion | Percent of dose recovered (%) |
|-------------|-----------|-------------------------------|
|             | Urine     | Feces | Total |
| 0 – 8       | 33.5      | 0.6   | 34.1  |
| 8 – 24      | 30.6      | 0.8   | 31.4  |
| Total       | 64.1      | 1.4   | 65.5  |

urine and feces from the \( ^{35} \text{S-FI6934} \) administered rabbits is shown in Table 3. The radioactivity excreted up to 8 hr after administration in the urine and feces was 33.5 and 0.6%, respectively. The cumulative excretion of radioactivity up to 24 hr was 64.1 and 1.4% in the urine and feces, respectively.

Metabolism in rats

Interaction of FI6934 with blood and tissue homogenates: In an attempt to determine the sites where FI6934 is hydrolysed in the animal body, we allowed FI6934 to make contact with the blood, the homogenates of kidneys or liver and then measured the rate of hydrolysis of FI6934 by bioassay. During 30 min of incubation at 37°C, FI6934 in the blood did not lose its physiological activity. On the other hand, FI6934 quickly decomposed when in contact with the kidneys or liver homogenate. The rate of inactivation of FI6934 was rapid, particularly in the kidneys homogenate.

Metabolites of \( ^{35} \text{S-FI6934} \) in the urine: \( ^{35} \text{S-FI6934} \) (specific radioactivity: 29 \( \mu \text{Ci/mg} \)) was injected into a male rat i.m. (500 \( \mu \text{g/kg} \)) and a 5 hr urine specimen was collected. The radioactivity recovered in the urine was found to be 69% of the dose given to the rat. The urine was applied directly on a filter paper and descending paper chromatography was carried out using the solvent at 80%. The paper chromatography gave four peaks of radioactivity, each of which was eluted with distilled water. The radioactivity was 1.3% for peak A, 63.1% for peak B, 27.7% for peak C and 7.9% for peak D. The spots of these metabolites were negative to ninhydrin indicating that their N-terminal pyrrolidone carboxylic acids were retained. They were also negative to the Ehrlich reagent, indicating the lack of tryptophane. These metabolites showed no contraction of the guinea pig gallbladders even when the molar concentration of the metabolites added was ten times higher than that of standard FI6934.

Separation of the main metabolites, F-I and F-II: The main radioactive peaks, B and C, on the paper chromatogram (the solvent 80%) were eluted and examined by paper chromatography (the solvent 1 : 1 : 1) and paper electrophoresis. Peaks B and C gave a single peak, respectively, in both methods, therefore, the metabolite in peaks B and C was denoted by F-I and F-II, respectively. The chromatographic pattern of F-I and F-II differed from that of \( ^{35} \text{S-Chym-I} \).

We were unable to analyse quantitatively the peaks A and D because they were so small in quantity.
The amounts of F-I and F-II separated from the urine of a rat, to which 125 µg of
$^{35}$S-F6934 was injected (500 µg/kg), were insufficient for their amino acid analysis. To
obtain sufficient amounts of metabolites to determine their amino acid composition, we
increased the dose of $^{35}$S-F6934 (specific radioactivity : 2 µCi/mg) tenfold (i.e. 5 mg/kg).
The amounts of F-I and F-II, however, hardly increased, while most of the radioactivity
excreted in the urine was the unchanged F6934.

Metabolites of $^{35}$S-Chym-I injected to rats: $^{35}$S-Chym-I (specific radioactivity, 0.4
µCi/mg) was injected to rats (1.6 mg/kg) and the 2 hr urine was collected to compare its
metabolites in rats with those of $^{35}$S-F6934. The urine was applied on paper chromato-
graphy using the solvent at 80%. As a result, we observed two main peaks, C-I and C-II,
and the $R_F$ value of C-I and C-II was identical to that of F-I and F-II, developed under
the same condition, respectively. (Table 4). Both spots of C-I and C-II showed negative
results to ninhydrin and to the Ehrlich reagent. These substances also carried no physio-
logical activity to induce the contraction of the gallbladder. We also found that the extent
of migration of C-I and C-II agreed well with that of F-I and F-II, respectively, in paper
chromatogram developed by the solvent 1 : 1 : 1 and paper electrophoresis (Table 4). From
these findings, it is considered that C-I and C-II are substances having a chemical structure
similar or identical to that of F-I and F-II, respectively.

In addition, it was found that, even when the dose of $^{35}$S-Chym-I was increased to 8
mg/kg, its main metabolites were still C-I and C-II, and 69% of the radioactivity admin-
istered was recovered in the urine collected in 8 hr after administration.

To analyse the amino acid composition of C-I and C-II, the 8 hr urine was collected
from rats to which $^{35}$S-Chym-I (specific radioactivity : 0.4 µCi/mg) was injected (8 mg/kg).
The urine thus obtained was concentrated about 20-fold and centrifuged. The supernatant
was subjected to paper chromatography and developed by the solvent 80%. C-I and C-II
were eluted with distilled water and the respective metabolite was subjected independently to
paper chromatography and developed with the solvent 1 : 1 : 1. C-I and C-II were eluted
again and then separated and purified by paper electrophoresis at pH 1.7. In paper electro-

| Samples   | Paper chromatography, $R_F$ | Paper Electrophoresis 1.7 Ecys | Reaction with ninhydrin | Reaction with the Ehrlich reagent | Bioassay Contraction | Dose (relative) |
|-----------|----------------------------|---------------------------------|------------------------|----------------------------------|----------------------|-----------------|
| 35S-Chym-I| 1.37                       | 0.48                            | 0.39                   | -                                | -                    | 10              |
| C-I       | 0.17                       | 0.31                            | 0.75                   | -                                | -                    | 10              |
| C-II      | 0.88                       | 0.98                            | 1.92                   | -                                | -                    | 10              |
| F-I       | 0.16                       | 0.31                            | 0.75                   | -                                | -                    | 10              |
| F-II      | 0.87                       | 0.92                            | 1.92                   | -                                | -                    | 10              |
| Sulfuric acid | /                           | /                               | 7.3                    | /                                | /                    | /               |

$R_F = \frac{\text{The migrated distance of a sample}}{\text{The migrated distance of F6934}}$
phoresis, these metabolites showed a single peak and the migrated distance of C-I and C-II thus purified was identical to that of F-I and F-II, respectively.

**Amino acid analysis of C-I and C-II:** The results of amino acid analysis of C-I and C-II are shown in Table 5. The molar ratio of the amino acids in C-I was as follows; Glu 2, Asp 1.2, Thr 0.9, Gly 0.5, Tyr 0.7 and Ser 0.2. The amount of C-I (0.050 µ mole) calculated from the results of amino acid analysis was in good agreement with that of C-I (0.053 µ mole) calculated from the specific radioactivity of C-I used for amino acid analysis. From this finding, C-I is considered to be a peptide derived from 35S-Chym-I.

On the other hand, the amount of C-II (0.002 µ mole) obtained from the results of amino acid analysis did not agree with that (0.048 µ mole) calculated from the specific radioactivity of C-II used for amino acid analysis. From this finding, C-II is not considered to be a peptide or an amino acid.

**DISCUSSION**

In order to trace the fate of FI6934 administered to animals, a radioactive tracer, 35S-labeled FI6934, was used. A biological assay technique based on the physiological activity of the peptide was also used to determine the concentration of FI6934 in the biological samples.

The effective range of dose of FI6934 to show its physiological activity is so low that it is impossible to trace its metabolism when it was administered to animals in a quantity within such dose. In the present study, we had to give doses far exceeding the threshold of physiological activity of the peptide. It should be noted that all the results described in this paper are related to the metabolism of FI6934 when administered at such large doses.

In the case that 35S-FI6934 is used as a tracer, one may consider the possibility that 35S-labeled sulfuric acid, liberated by hydrolysis of the tyrosylsulfate group of the peptide, or 35S-labeled substance, produced by re-arrangement of 35S-labeled sulfuric ion, would be traced. In the present study, however, it was proved that free 35S-labeled sulfuric acid was undetectable in the urine from the 35S-FI6934 administered rats, and that, at least, about 45% of the radioactive substances recovered in the 5 hr urine are the 35S-labeled...
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peptides which are metabolites of 35S-F16934.

The differences in physiological actions of F16934 in such animals as rabbits, cats, dogs and rats, etc., have been reported (3), and suggest that there may also be differences concerned with the fate of the peptide in an animal body between species. In this study, several differences in absorption and excretion of the peptide were observed between rats and rabbits. The blood level of radioactivity in rats decreased rapidly immediately after administration of 35S-F16934, whereas in rabbits it changed rather slowly showing a peak at 15 min after administration. In rats, the blood level of radioactivity increased slightly 6 hr after injection, suggesting a reabsorption of biliary excreted radioactive compounds through the intestine. By contrast, such a phenomenon was not observed to occur in rabbits, which can be explained by the fact that the biliary excretion of radioactivity in rabbits is much smaller in quantity and slower than in rats. When 280 and 380 μg of F16934 per kg was injected to rats and rabbits, respectively, blood level of F16934 determined physiologically was lowered rapidly. On the other hand, in vitro reaction of F16934 with rat blood induced no decrease in its physiological activity, whereas F16934 brought into contact with the kidneys or liver homogenates of rats quickly lost its physiological activity. These facts indicate that the peptide is not hydrolysed in the blood but is decomposed by the enzymes in such organs as kidneys and liver. Since the peptide is excreted in the bile, it is presumably hydrolyzed with chymotrypsin in the small intestines. However, the amount of radioactivity excreted in the small intestines within 8 hr after its administration was about 10% of the dose administered, while the amount of radioactivity excreted in the 8 hr urine reached 70 to 80% of the dose administered. In addition, the urine showed no physiological activity of F16934 whatever (3). Such being the case, it is assumed that the metabolism of the peptide occurred for the most part in such organs as kidneys and liver. It is considered that the C-terminal tetrapeptide amide of F16934 is indispensable for the peptide to exert its physiological activities (12). Therefore, it is thought that the physiological activities of the peptide are lost by the hydrolysis of its Gly-Try bond.

The radioactivity of 35S-F16934 administered is mainly excreted in the urine through the kidneys in both rats and rabbits. Small amounts of radioactivity detected in the feces are considered to be derived from some of the biliary excreted radioactive substances, although contamination of the samples with the urine may also be responsible for a part of the radioactivity.

In rats, the excretion rate of radioactivity in the bile was rapid, reaching 8.76 per cent of the dose administered in 1 hr after administration, and it was demonstrated in the biological assay that most of the radioactive substances in the bile collected within the first 2 hr had the physiological activity of F16934. In contrast, the radioactive substances excreted into the bile of rabbits had no physiological activity. If the radioactive substance in the bile of rabbits is assumed to be unchanged F16934, its concentration would be estimated to be 0.9 μg/ml at the maximum, and the concentration of F16934 in the sample for the biological assay would be 1.8 × 10^{-3} g/ml (1:50 dilution, see the method of experiments.) Under such a condition, gallbladders are expected to show contraction responses in the
sample solution, since the threshold of FI6934 to induce the gallbladder contraction is about 10^{-9} g/ml. Accordingly, most of the radioactive substances in the bile of rabbits are considered to have been metabolized to lose the physiological activity. Time dependent changes in the bilirubin index and bile secretion rate were measured in rabbits simultaneously with the assays of radioactivity and physiological activity. On administering FI6934 to rabbits, the bile secretion rate was not increased, but rather decreased. The bilirubin index, an indicator of the content of bile pigments, showed a transient decrease after injection of FI6934 and then increased to a level higher than that measured before administration. The relation between these phenomena and the physiological action of FI6934 cannot be explained, since the administered dose of FI6934 is 380 \mu g per kg, which is much higher than the physiologically effective concentration of the peptide.

Although the effect of FI6934 on the secretion rate of the pancreatic fluid and protein was also examined together with the measurement of the radioactivity in the fluid, it is difficult to explain the relationship between the phenomena observed and the physiological activity of FI6934, for the same reason as in the case of the biliary excretion. The secretion of the pancreatic fluid was temporarily decreased by administration of FI6934, and increased again 3 hr later. The protein content in the pancreatic fluid also showed a similar temporary decrease, and increased again 3 hr after administration. These phenomena suggest that the secretory function of the pancreas once had been inhibited by the injection of a large dose of FI6934 (18), and was restored by decomposition of the peptide after a certain period.

In the experiments in rats, the dense distribution of radioactivity in such organs as the liver, pancreas and gastro-intestinal tract, was clearly shown. In another experiment we also examined the distribution of ^{35}S-FI6934 in mice using the technique of whole body autoradiography, and observed dense distribution of radioactivity in the gallbladder (19). These results suggest that the peptide affects these organs directly. The radioactivity in the intestine is considered to be derived from that excreted together with the bile.

We found at least four different metabolites of ^{35}S-FI6934 in the rat urine. The authors first gave large doses (5 mg/kg) of ^{35}S-FI6934 to the rats in an attempt to obtain F-I and F-II in quantities sufficient for amino acid analysis, but no significant increase in these metabolites was observed, while unchanged ^{35}S-FI6934 was excreted for the most part in the urine. Such being the case, we were unable to make amino acid analysis of these metabolites.

Since the metabolite F-I possessed radioactivity, it is considered to have tyrosil-sulfate moiety. From the fact that F-I was negative to ninhydrin, it is suggested that pyrrolidin carboxylic acid was retained at N-terminal. From the facts that it gave negative results to bioassay and to the Ehrlich reagent, it was presumed that the peptide lacked tryptophan and four or more amino acids from its C-terminal. On the other hand, since the C-terminal of FI6934 is an amido structure (12), it is presumed that the hydrolysis of C-terminal of the peptide by such enzymes as carboxypeptidase would be difficult, while the peptide bond between tryptophan and methionine would be easily decomposed with such enzymes as chymotrypsin. Therefore, when metabolized in an animal body, there is a great possibility
that the peptide is split at the position between tryptophan and methionine. Considering the fact, however, that the most of the radioactivity excreted was the unchanged peptide, (large dose of ^3^S-FI6934), there is the possibility that the hydrolizing reaction of the Trp-Met bond is the rate determining step of the metabolism of FI6934. This being so, if ^3^S-FI6934 is treated with chymotrypsin to hydrolyse the Trp-Met bond and ^3^S-Chym-I thus obtained is administered in large doses, it is expected that large quantities of F-I or substances similar to F-I would be produced.

The structure of Chym-I:

\[
\text{Pyr-Gln-Asp-Tyr-Thr-Gly-Trp} \quad \text{SO}_3\text{H}
\]

On such a working hypothesis we administered ^3^S-Chym-I to rats and examined the metabolites in the urine. As a result the main metabolites C-I and C-II were obtained and they increased by increasing the dose of ^3^S-Chym-I. Moreover, C-I and C-II was shown to be identical or very similar to F-I and F-II, respectively, in paper electrophoresis and paper chromatography. C-I and C-II were obtained in quantities sufficient for amino acid analysis.

The results of the amino acid analysis of C-I showed that the molar ratio of the amino acids was Glu (2), Asp (1.2), Tyr (0.7), Thr (0.9), Gly (0.5) and Ser (0.2). Although the origin of the serine was unknown, from the fact that it was so small in quantity and that serine was not found in such peptides as FI6934 and Chym-I which had not passed through the body of a rat (see Table V), it was considered that C-I was probably contaminated with small amounts of substances containing serine while it was passing through the body of the rat. Amino acids other than serine were the constituents of FI6934 and their molar ratios were also the same as those assumed from the structure of FI6934 or Chym-I. Since, C-I showed negative results in the ninhydrin test, its N-terminal is considered to be pyrrolidon carboxylic acid. The fact that C-I showed negative results to the Ehrlich reagent indicates that there was no tryptophan in C-I. (Chym-I showed positive results to the Ehrlich reagent.) From the above findings, C-I is considered to be a peptide which was produced by the separation of tryptophan from C-terminal of Chym-I, and its primary structure is considered to be as follows:

Estimate structure of C-I

\[
\text{Pyr-Gln (or Glu)-Asp-Tyr-Thr-Gly} \quad \text{SO}_3\text{H}
\]

Although glutamine of C-I might be transformed into glutamic acid by the action of deaminase in the animal body, we were unable to examine this possibility because the amounts of C-I were insufficient.

According to the results of amino acid analysis (see Table V), C-II is not considered to be a peptide or an amino acid. In addition, C-II migrates much more rapidly than ^3^S-sulfuric acid in paper electrophoresis (see Table IV). Therefore, C-II was considered...
to be hot a free sulfuric acid but a substance which was formed by the transfer of $^{35}$S-sulfuric acid from $^{35}$S-Chym-I to another substance. Since C-II and F-II are nearly the same substance according to paper chromatography and electrophoresis, F-II was also considered to be a substance formed by the transfer of $^{35}$S-sulfuric acid of $^{35}$S-F16934 to a substance other than a peptide.

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