

Studies on the Metabolites in Urine and Feces of Rat after Oral Administration of Radioactive Pantethine¹⁾

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This is the first report describing identification of PaSSO₃H in nature and P-PaSSO₃H in mammals.

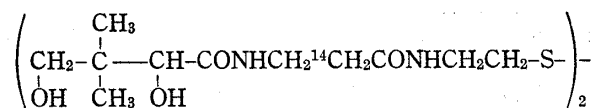
Rats fed with solid feed containing PaA excreted PaA and P-PaA in urine, and PaA, P-PaA and PaSS in feces. When ¹⁴C-PaSS was orally administered to the rats, 15—35% of the radioactivity was excreted in a week. The amount of radioactivity excreted into urine was about double to that excreted into feces. The metabolites identified are: PaA, PaSS, P-PaA and/or PaSSO₃H and P-PaSSO₃H from urine, and PaA, PaSS, PaSSO₃H, P-PaA from feces.

The possible formation mechanisms of PaSSO₃H and P-PaSSO₃H in rat were discussed.

PaSS was discovered by Snell, *et al.*³⁾ as a *Lactobacillus burgaricus* factor (LBF) along with its reduced form, PaSH. In later investigation, PaSH moiety was proved to be in common with CoA,⁴⁾ ACP⁵⁾ and fatty acid synthetases from yeast^{6a)} and pigeon liver.^{6b)} Actually, radioactive PaSS was incorporated into P-PaSH, CoA and ACP-like substance in *Bifidobacterium bifidum* N4.⁷⁾ PaSS was required by many lactic acid bacteria,^{8,9)} and showed high growth activity to all the strains of *B. bifidum* tested,¹⁰⁾ while about half of them did not grow with PaA.^{10,11)} Based on the high activity of PaSS to *B. bifidum*, Ohta, *et al.*¹²⁾ orally administered it to infants in order to investigate its proliferative effect on *B. bifidum in vivo*. How-

- 1) This work was presented at The 92th Annual Meeting of Pharmaceutical Society of Japan, Osaka, April 1972. The abbreviations used in this paper are: PaA, pantothenic acid; P-PaA, 4'-phosphopantothenic acid; PaCySH, N-pantothenyl-L-cysteine; P-PaCySH, N-pantothenyl-L-cysteine-4'-phosphate; P-PaCySS, N,N'-di-pantothenyl-L-cystine-4',4''-diphosphate; PaSH, pantetheine; PaSS, pantethine; PaSHZ, pantetheine thiazoline; P-PaSH, 4'-phosphopantetheine; P-PaSS, 4',4''-diphosphopantetheine; PaSSO₃H, pantetheine-S-sulfonic acid; P-PaSSO₃H, 4'-phosphopantetheine-S-sulfonic acid; DP-CoA, 3'-dephospho-coenzyme A; CoA, coenzyme A; DP-CoASSO₃H, 3'-dephospho-coenzyme A-S-sulfonic acid; CoASSO₃H, coenzyme A-S-sulfonic acid; CySSO₃H, cysteine-S-sulfonic acid; GSSO₃H, glutathione-S-sulfonic acid; ACP, acyl carrier protein; MeOH, methyl alcohol; PC, paper chromatography and PEP, paper electrophoresis.
- 2) Location: Hongo-7-3-1, Bunkyo-ku, Tokyo.
- 3) E.E. Snell, G.M. Brown, V.J. Peters, J.A. Craig, E.L. Wittle, J.A. Moore, V.M. McGlohon, and O.D. Bird, *J. Am. Chem. Soc.*, **72**, 5349 (1950).
- 4) J. Baddiley, E.M. Thain, G.D. Novelli, and F. Lipmann, *Nature*, **171**, 76 (1953); J.G. Moffatt and H.G. Khorana, *J. Am. Chem. Soc.*, **81**, 1265 (1959).
- 5) T.C. Vanaman, S.J. Wakil, and R.L. Hill, *J. Biol. Chem.*, **243**, 6420 (1968).
- 6) a) F. Lynen, *Biochem. J.*, **102**, 381 (1967); b) C.J. Chesterton, P.H.W. Butterworth, and J.W. Porter, *Arch. Biochem. Biophys.*, **126**, 864 (1968).
- 7) Z. Tamura, H. Nakamura, and T. Nishikawa, Abstracts of Papers, Symposium on Molecular Biology about Microorganisms and Its Application to Pharmaceutical Region, Pharmaceutical Society of Japan, Kanazawa, August, 1971, p. 79.
- 8) W.L. Williams, E. Hoff-Jørgensen, and E.E. Snell, *J. Biol. Chem.*, **177**, 933 (1949).
- 9) E. Kitay and E.E. Snell, *J. Bacteriol.*, **60**, 49 (1950).
- 10) A. Sakashita and Y. Ohshima, *Vitamins (Japan)*, **42**, 65 (1970).
- 11) M. Yoshioka, S. Yoshioka, Z. Tamura, and K. Ohta, *Japan. J. Microbiol.*, **12**, 395 (1968).
- 12) K. Ohta, N. Homma, T. Koyama, Y. Ichihashi, T. Niki, T. Urano, A. Sakashita, and Y. Ohshima, *Acta Paediatrica Japonica*, **75**, 1003 (1971).

ever, the fate of PaSS in mammals has not been known except its increasing effect of CoA level in liver,¹³⁾ kidney¹³⁾, and testicle.¹⁴⁾ Therefore, we investigated the metabolites excreted in feces and urine of rats orally administered ¹⁴C-PaSS.



Experimental

1. Chemicals and Materials—Radiochromatographically and bioautographically pure radioactive PaSS (¹⁴C-PaSS), synthesized by Nishikawa, *et al.*⁷⁾ from calcium D-pantothenate-2-¹⁴C, was used in this experiment. The specific activity was 2.53 mCi/mmmole. P-PaA, PaSS, P-PaSH, CoA, DP-CoA and PaSSO₃H were kindly provided by Daiichi Seiyaku Co., Ltd., Tokyo. P-PaSSO₃H,¹⁵⁾ DP-CoASSO₃H¹⁶⁾ and CoASSO₃H¹⁶⁾ were synthesized by the sulfitolysis of P-PaSS, DP-CoA and CoA, respectively, and purified by QAE-Sephadex A-25 column chromatography (OH⁻, 0.9 × 11 cm) and PC, obtaining bioautographically pure compounds (see sections 5 and 6). Calcium D-pantothenate was purchased from Daiichi Pure Chemicals Co., Ltd., Tokyo. Other chemicals and solvents were all guaranteed grade. QAE-Sephadex A-25 (OH⁻) was prepared by washing the commercial resin (Cl⁻) with 1 N NaOH and then water. For PC and PEP, Toyo Filter Paper No. 514 was directly used.

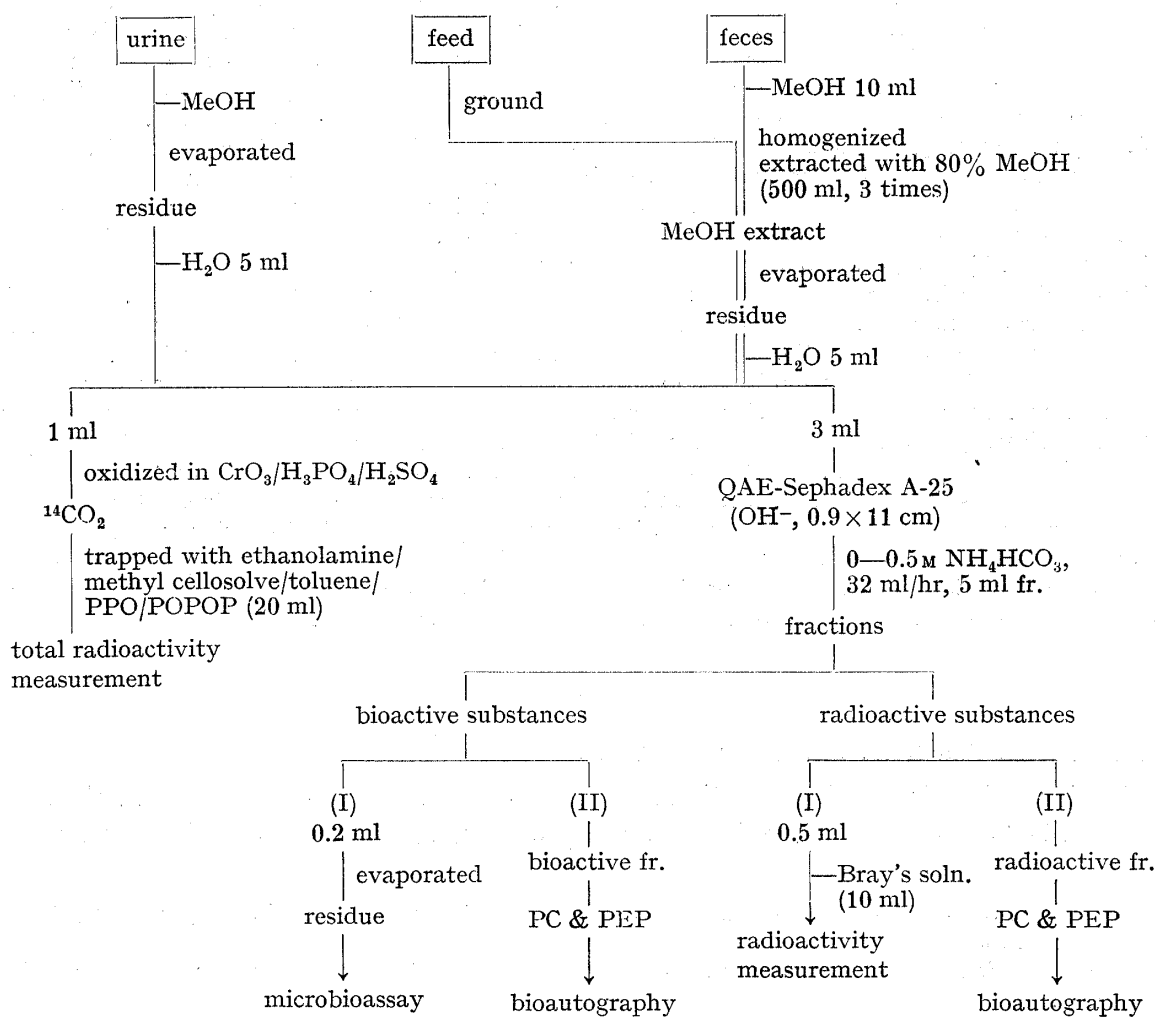


Chart 1. Analytical Procedures for Specimens and Feed

13) E. Mascitelli-Coriandoli, R. Boldrini, and C. Citterio, *Naturwissenschaften*, **46**, 111 (1959).

14) E. Mascitelli-Coriandoli and C. Citterio, *Naturwissenschaften*, **47**, 183 (1960).

15) M. Yoshioka and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **19**, 178 (1971).

16) M. Yoshioka and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **19**, 186 (1971).

2. Administration of ^{14}C -PaSS to Rats—Three Wistar male rats, weighing 340–354 g, were fed with CE-2¹⁷⁾ (Japan CLEA Co., Ltd., Tokyo). After 7 days' feeding, 2 ml of aqueous solution of PaSS (500 μg , 1.24×10^4 cpm) was once administered to the rats using a stomach cannula and urine and feces were collected every 24 or 48 hr; urine was collected over 2 ml of MeOH. Control specimens were obtained from rats without administration of PaSS.

3. Pretreatment of Specimens—Pretreatment and successive procedures for specimens and feed are summarized in Chart 1.

Urine: Urine was evaporated to dryness¹⁸⁾ after addition of equal volume of MeOH.

Feces: Feces were crushed with 10 ml of MeOH and extracted three times with each 500 ml of 80% MeOH. The extracts were combined and evaporated to dryness.

Feed: Five pieces of feed were crushed, ground and extracted three times with each 500 ml of 80% MeOH. The extracts were combined and evaporated to dryness.

4. Measurement of Total Radioactivity in Specimens—The evaporated specimens were mixed with 5 ml of water and a 1 ml-aliquot was oxidized by the mixture of CrO_3 (25 g), H_3PO_4 (167 ml) and H_2SO_4 (333 ml).¹⁹⁾ The apparatus for wet carbon combustion and absorbent of liberated $^{14}\text{CO}_2$ were essentially identical to those described by Kametani, *et al.*²⁰⁾ excepting the following modifications; the reaction was performed in a 50-ml round-bottomed, three-necked flask, heating time was 20 min and the successive passage of nitrogen stream was continued for 10 min. The trapped radioactivity was measured with Packard Scintillation Spectrometer Model 3214.

5. OAE-Sephadex Column Chromatography—Each 3 ml of the turbid solution obtained in the preceding section 4 was charged onto a column of QAE-Sephadex A-25 (OH^- , 0.9×11 cm) and gradient elution was performed with 300 ml of 0–0.5 M NH_4HCO_3 . Sixty fractions of 5 ml were collected with a constant flow rate of 32 ml/hr. The 0.5 ml-aliquot of the fractions was added to 10 ml of Bray's solution²¹⁾ and counted for radioactivity for 10 min with the apparatus described above. From each fraction obtained from feed and control specimens, a 0.2 ml-aliquot was taken and lyophilized three times by adding 0.2 ml of water to remove NH_4HCO_3 and assayed with *B. bifidum* S4²²⁾ as described in section 7.

6. Paper Chromatography and Paper Electrophoresis—The radioactive or bioactive fractions obtained by the column chromatography were evaporated to dryness repeatedly with the addition of water to remove NH_4HCO_3 , and dissolved in a drop of water. PC was performed with an ascending method for 3–4 hr with a solvent system of *n*-butanol/acetic acid/water (5:2:3, by vol.). Detections were performed by bioautography²³⁾ with *B. bifidum* S4 without the diffusion barrier device.

Two kinds of buffer were used for PEP: 1 M HCOOH (pH 2) for 2 hr at 11.8 v/cm and 0.1 M NH_4HCO_3 (pH 8) for 2 hr at 11.8 v/cm. The apparatuses used are SJ-1050 (A) (Mitsumi Scientific Ind., Co., Ltd., Tokyo) and Type C (Toyo Roshi Co., Ltd., Tokyo) for the former and the latter, respectively. Detections were performed by bioautography and by the following procedure. The paper electrophorogram was cut into pieces of 1 cm-width and the radioactivity was measured in 10 ml of the Bray's solution²¹⁾ as described above.

7. Microbioassay—*B. bifidum* S4 was cultivated for 18 hr at 37° in an atmosphere of N_2 - CO_2 (9:1) in 2 ml of the preculture medium.¹¹⁾ The cells were harvested, washed three times with 0.85% saline supplemented with L-ascorbic acid (25 mg/ml) and suspended in 2 ml of the same saline. One drop of the suspension was diluted with 10 ml of the same saline and one drop of the resulted solution was inoculated into the fraction of QAE-Sephadex column chromatography added with 3 ml of the basal medium.¹¹⁾ After 48 hr of anaerobic cultivation at 37°, the acid produced was titrated with 0.2 N NaOH after addition of 0.1 ml of ethanolic mixed solution of neutral red (1%) and bromothymol blue (0.5%).

Result

Excretion of Radioactivity into Urine and Feces

As shown in Fig. 1, the excretion rate of radioactivity became slow from the second day, and 15–35% of administered radioactivity was excreted in a week, indicating individual

17) The average daily ingestion of feed and PaA were 20 g and 480 μg per capial, respectively (CE-2 contains PaA 24 $\mu\text{g}/\text{g}$).

18) The evaporation was performed with a rotary evaporator at below 40° *in vacuo* throughout this investigation.

19) D.D. Van Slyke and J. Folch, *J. Biol. Chem.*, **136**, 509 (1940).

20) K. Kametani, Y. Inoue, and K. Maruyama, *Radioisotopes*, **16**, 41 (1967).

21) G.A. Bray, *Anal. Biochem.*, **1**, 279 (1960).

22) H. Nakamura and Z. Tamura, *Japan. J. Microbiol.*, **16**, 239 (1972); The strain showed growth activity to PaA, P-PaA, PaCySH, P-PaCySS, PaSS, PaSHZ, P-PaSH, DP-CoA, CoA, PaSSO₃H, P-PaSSO₃H, DP-CoASSO₃H and CoASSO₃H.

23) H. Nakamura, K. Samejima, and Z. Tamura, *Japan. J. Microbiol.*, **14**, 9 (1970).

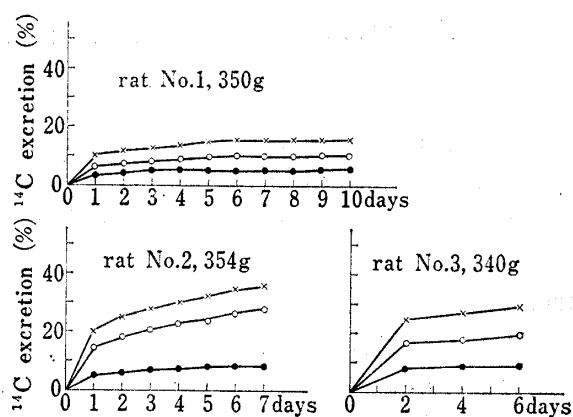


Fig. 1. Cumulative Excretion of Radioactivity into Urine and Feces after Oral Administration of ^{14}C -PaSS

○: urine ●: feces ×: urine + feces

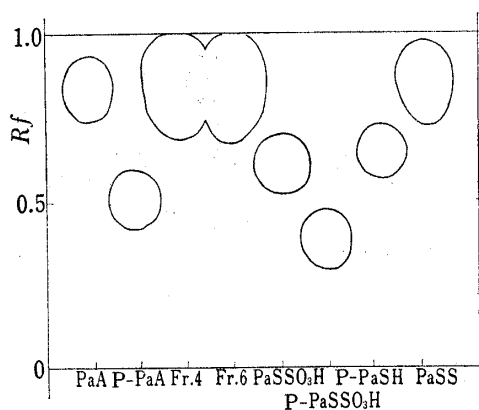


Fig. 3. Bioautogram of a Paper Chromatogram of the Fractions obtained in Fig. 2

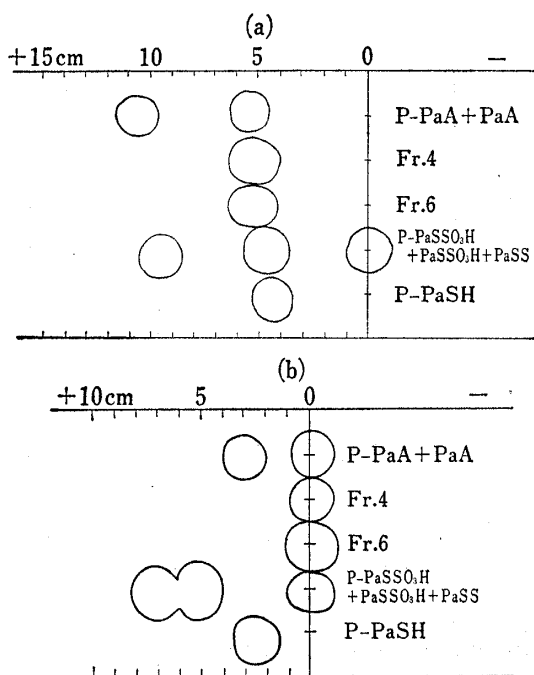


Fig. 4. Bioautograms of Paper Electropherograms at pH 8 (a) and pH 2 (b) of the Fractions obtained in Fig. 2

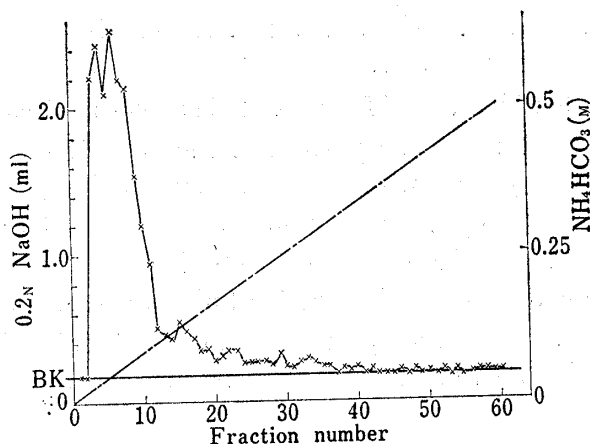


Fig. 2. Elution Pattern from a QAE-Sephadex Column of The 80% MeOH Extract of Feed

BK: inoculated blank

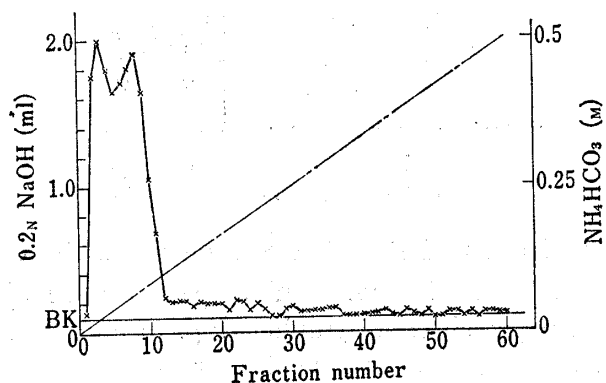


Fig. 5. Elution Pattern from a QAE-Sephadex Column of the Control Urine

BK: inoculated blank

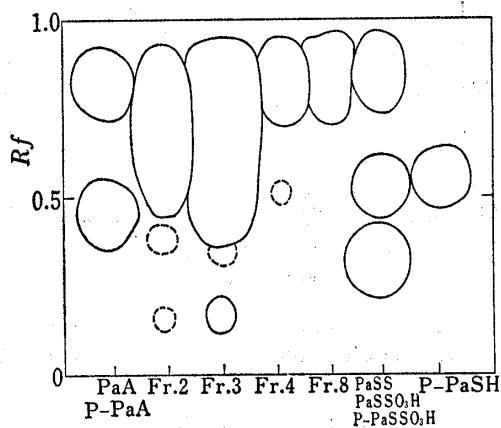


Fig. 6. Bioautogram of a Paper Chromatogram of The Fractions obtained in Fig. 5

deviation. The amount of radioactivity excreted into urine was about double to that excreted into feces.

Identification of Bioactive Substances in Feed

The elution pattern of the 80% MeOH extracts of feed from a QAE-Sephadex column (OH^-) is shown in Fig. 2. Two major peaks whose tops were located in fraction 4 and 6 respectively, were obtained. However, these were proved to be substantially composed of only PaA by PC (Fig. 3) and by PEP (Fig. 4).

Identification of Bioactive Substances in the Control Urine

The elution pattern is shown in Fig. 5. PC (Fig. 6) and PEP (Fig. 7) indicate the presence of P-PaA in fractions 2—4 besides PaA, which was the sole bioactive substance in fraction 8.

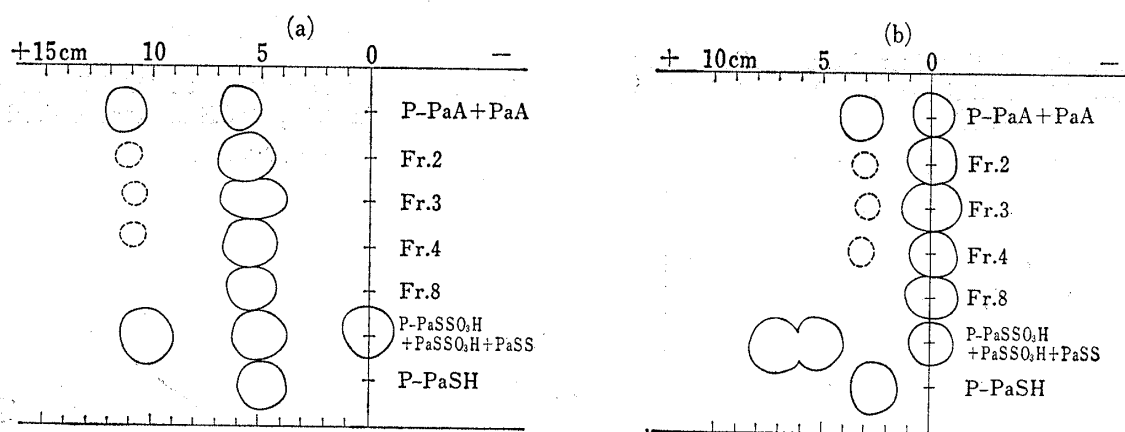


Fig. 7. Bioautograms of Paper Electrophorograms at pH 8 (a) and pH 2 (b) of The Fractions obtained in Fig. 5

Identification of Bioactive Substances in the Control Feces

As shown in Fig. 8, minor peak I and major peak II were obtained. It was concluded from paper chromatographic (Fig. 9) and electrophoretic behaviours (Fig. 10) that the bioactive substance of the peak I (fraction 3) was PaSS and those of the peak II (fractions 7, 9, and 11) were PaA and trace P-PaA.

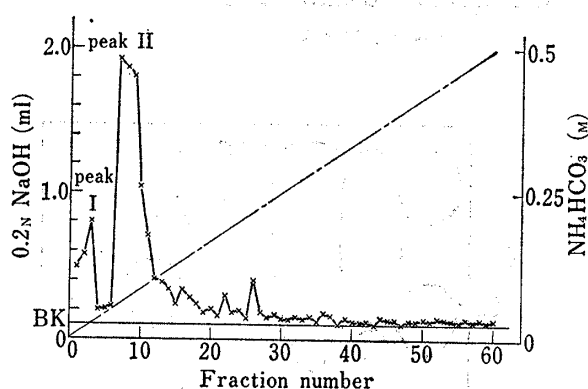


Fig. 8. Elution Pattern from a QAE-Sephadex Column of The 80% MeOH Extract of The Control Feces

BK: inoculated blank

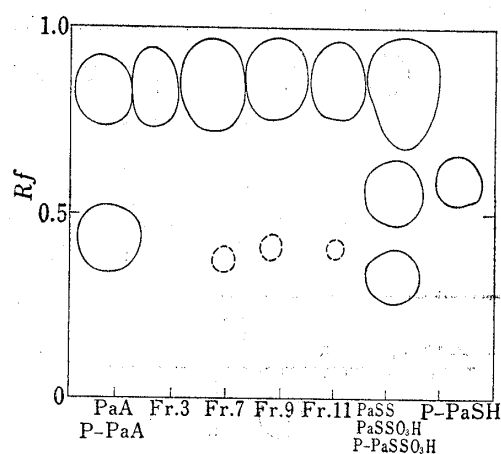


Fig. 9. Bioautogram of a Paper Chromatogram of the Fractions obtained in Fig. 8

Identification of Metabolites in Urine of Rats Administered with ^{14}C -PaSS

As shown in Fig. 11, the majority of the radioactivity was eluted from the column with low concentration of NH_4HCO_3 . It was proved by PC (Fig. 12) and PEP (Fig. 13) that fraction 2 contained PaSS and fraction 7 contained PaA, P-PaSSO₃H and P-PaA and/or PaSSO₃H.

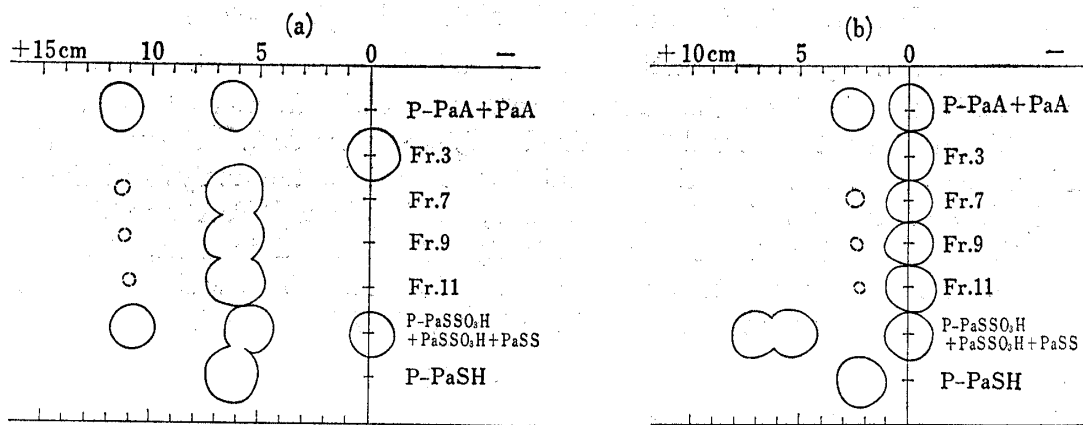


Fig. 10. Bioautograms of Paper Electrophorograms at pH 8 (a) and pH 2 (b) of The Fractions obtained in Fig. 8

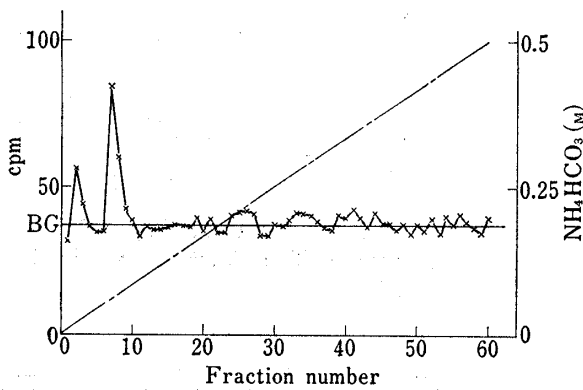


Fig. 11. Elution Pattern from a QAE-Sephadex Column of Urine of Rat administered with ¹⁴C-PaSS

BG: background

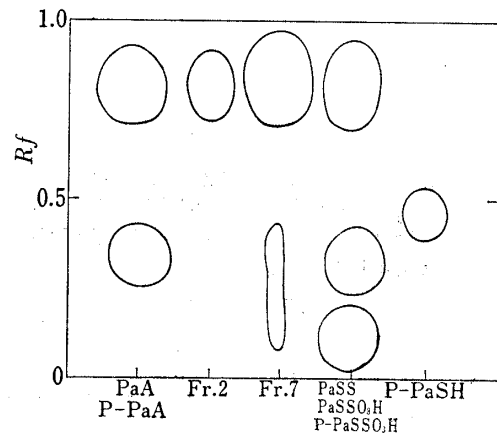


Fig. 12. Bioautogram of a Paper Chromatogram of The Fractions obtained in Fig. 11

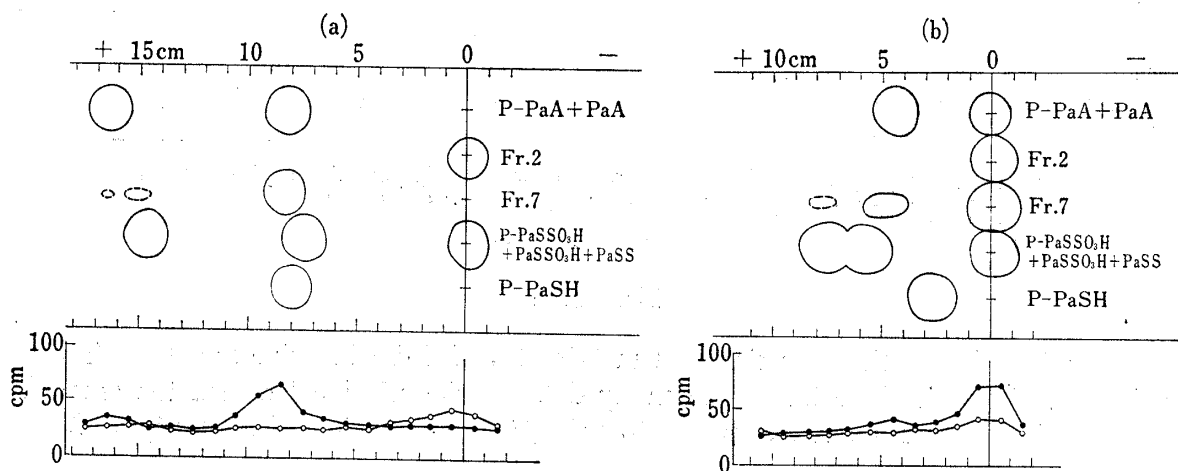


Fig. 13. Bio-(upper) and Radioautograms (lower) of Paper Electrophorograms at pH 8 (a) and pH 2 (b) of The Fractions obtained in Fig. 11

○—○: Fr. 2, ●—●: Fr. 7

Identification of Metabolites in Feces of Rats Administered with ^{14}C -PaSS

As shown in Fig. 14, two radioactive peaks (I and II) were obtained. The identification of the radioactive metabolites in the peak I was carried out with fractions 2 and 3. As shown in Figures 15 and 16, fractions 2 and 3 contained PaSS and trace PaA. The radioactivity was detected in PaSS (Fig. 16). Fractions 5 and 6 were used for the analysis of the peak II. As already shown in Figures 15 and 16, PaA, PaSS, PaSSO₃H and P-PaA were bioautographically identified in fraction 6. The radioactivity was detected in PaA, PaSS and also in PaSSO₃H. The similar results were obtained with fraction 5 as with fraction 6.

PaA derivatives in specimens and feed are summarized in Table I.

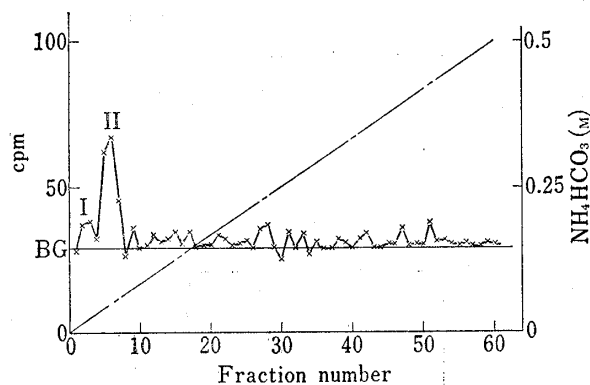


Fig. 14. Elution Pattern from a QAE-Sephadex Column of The 80% MeOH Extract of Feces of Rat administered with ^{14}C -PaSS

BG: Background

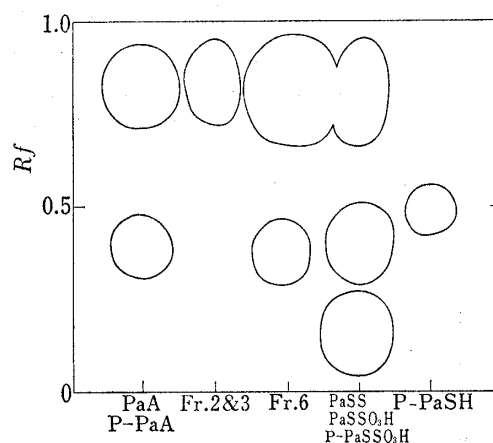


Fig. 15. Bioautogram of a Paper Chromatogram of The Fractions obtained in Fig. 14

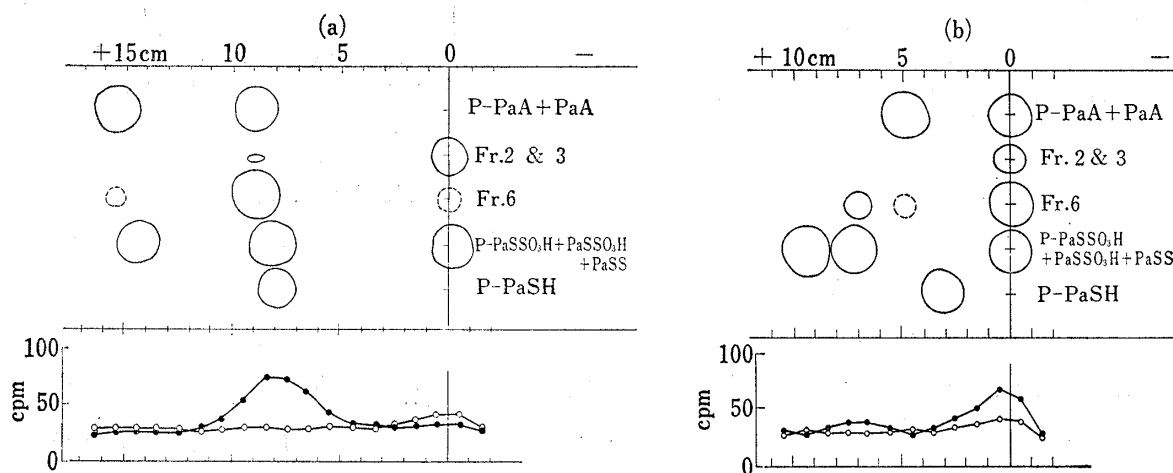


Fig. 16. Bio-(upper) and Radioautograms (lower) of Paper Electrophorograms at pH 8 (a) and pH 2 (b) of The Fractions obtained in Fig. 14

—○—: Fr. 2 & 3 —●—: Fr. 6

Discussion

To our knowledge, the excretion and metabolites of PaSS in urine and feces have not been known. Though there have been many data on the relation between the amount of PaA ingested and its amount excreted into urine,²⁴ the excretion of bound-type PaA (P-

24) R.E. Hodges, M.A. Ohlson, and W.B. Bean, *J. Clin. Invest.*, **37**, 1642 (1958); Y. Yoshitoshi, N. Shibata, K. Akazawa, and S. Yamashita, *Eiyo To Shokuryo*, **17**, 405 (1965); J.K. Pace, L.B. Stier, D.D. Taylor, and P.S. Goodman, *J. Nutr.*, **74**, 345 (1961).

TABLE II. Formation of PaSSO₃H from PaSS in Rat^{a)}

Specimen	Treatment			
	Not treated (a)	Boiled (b)	Filtered (c)	Boiled and filtered (d)
Urine	+	tr	—	tr
Fecal extract	++	++	++	++

a) The formation of PaSSO₃H was bioautographically evaluated and abbreviated as follows: —, no formation; tr, trace; +, conversion of 10–20% of PaSS into PaSSO₃H; ++, conversion of 20–40% of PaSS into PaSSO₃H.

PaA, P-PaCySH, PaSH, P-PaSH, DP-CoA, CoA, *etc.*) into urine has never been reported. Bigler, *et al.*²⁵⁾ described that any bound-type PaA was not found in human urine after intravenous injection of CoA.

As shown in Table I, the control rats, fed with feed containing only PaA as PaA derivatives, excreted P-PaA beside PaA into urine and feces. It is not certain whether the P-PaA is a product from phosphorylation of PaA²⁶⁾ or one from degradation of P-PaSH, CoA and other P-PaSH-containing substances. Moreover, PaSS was identified from the feces of the control rats, which indicates first incorporation of PaA into CoA or its higher precursors followed by degradation of them to PaSH and its oxidation to PaSS.

Even 7 days after the administration of ¹⁴C-PaSS, the excretion of radioactivity into urine and feces was less than 40% of the dose administered (Fig. 1). This low excretion of radioactivity would indicate incorporation of PaSS into CoA which stays long and plays various physiological functions in the living body. In fact, the increase of CoA level in liver¹³⁾ and kidney¹³⁾ in rat and testicle¹⁴⁾ in mouse after administration of PaSS was reported. PaA was the main metabolite of ¹⁴C-PaSS in urine and feces. The excretion of ¹⁴C-PaSS administered in urine suggests the possibility of the passage of PaSS as an intact form from mucosal to serosal side of the gut.

There are only several reports of the occurrence of S-sulfonic acids (RSSO₃H) in nature. GSSO₃H has been isolated from the calf lens²⁷⁾ and from the rat small intestine.²⁸⁾ Since Nakamura, *et al.*²⁹⁾ identified CySSO₃H as an intermediate in biosynthesis of cysteine in *Aspergillus nidulans*, CySSO₃H has been detected in the urine of rats,³⁰⁾ of blotchet Kenya genets³¹⁾ and of a patient with sulfite oxidase deficiency.³²⁾ Of considerable interest is the formation of CySSO₃H from cystine by molds.³³⁾ Yoshioka, *et al.* isolated P-PaSSO₃H and DP-CoASSO₃H from the carrot roots.^{15,16)} In this investigation, we identified P-PaSSO₃H in the urine and PaSSO₃H in the feces and probably in the urine of the rats after oral administration of PaSS. This is the first report of the identification of PaSSO₃H in nature, and of P-PaSSO₃H in mammals. Since these two compounds were not found in the control rats, it is clear that they have been derived from PaSS administered.

About the biosynthesis of S-sulfonic acid-type PaSH related compounds, we have no information. In this investigation, PaSSO₃H was found in the feces more than in the urine, suggesting the participation of the microorganisms in its formation. However, contribution

25) F. Bigler and H. Thölen, *Experientia*, **22**, 321 (1966).

26) G.M. Brown, *J. Biol. Chem.*, **234**, 370 (1959); Y. Abiko, *J. Biochem.* (Tokyo), **61**, 290 (1967).

27) S.G. Waley, *Biochem. J.*, **71**, 132 (1959).

28) H.C. Robinson and C.A. Pasternak, *Biochem. J.*, **93**, 487 (1964).

29) T. Nakamura and R. Sato, *Nature*, **185**, 163 (1960).

30) D. Cavallini, C. DeMarco, B. Mondovi, and L. Tentori, *J. Chromatog.*, **3**, 20 (1960).

31) J.C. Crawhall and S. Segal, *Nature*, **208**, 1320 (1965).

32) F. Irreverre, S.H. Mudd, W.D. Heizer, and L. Laster, *Biochem. Med.*, **1**, 187 (1967).

33) I. Matsunaga, *Japan. J. Microbiol.*, **14**, 508 (1970).

of non-enzymatic reactions such as sulfitolysis of PaSS or the reaction between PaSH and thiosulfate, analogous to the chemical formation of CySSO_3H ,³⁴⁾ can not be excluded. Therefore, PaSS was incubated with the urine or the saline extract of the feces of the control rats, with or without various treatments (Chart 2). As shown in Table II, in the fecal specimens the formation of PaSSO_3H from PaSS was observed in all treatments, while PaSSO_3H was scarcely formed through the incubation with the urine after sterilization. These results indicate the predominancy of the non-enzymatic formation of PaSSO_3H in feces and the participation of microorganisms in urine of rats. Such participation of microorganisms in formation of CySSO_3H in urines of animals^{30 31)} during their collection will be possible.

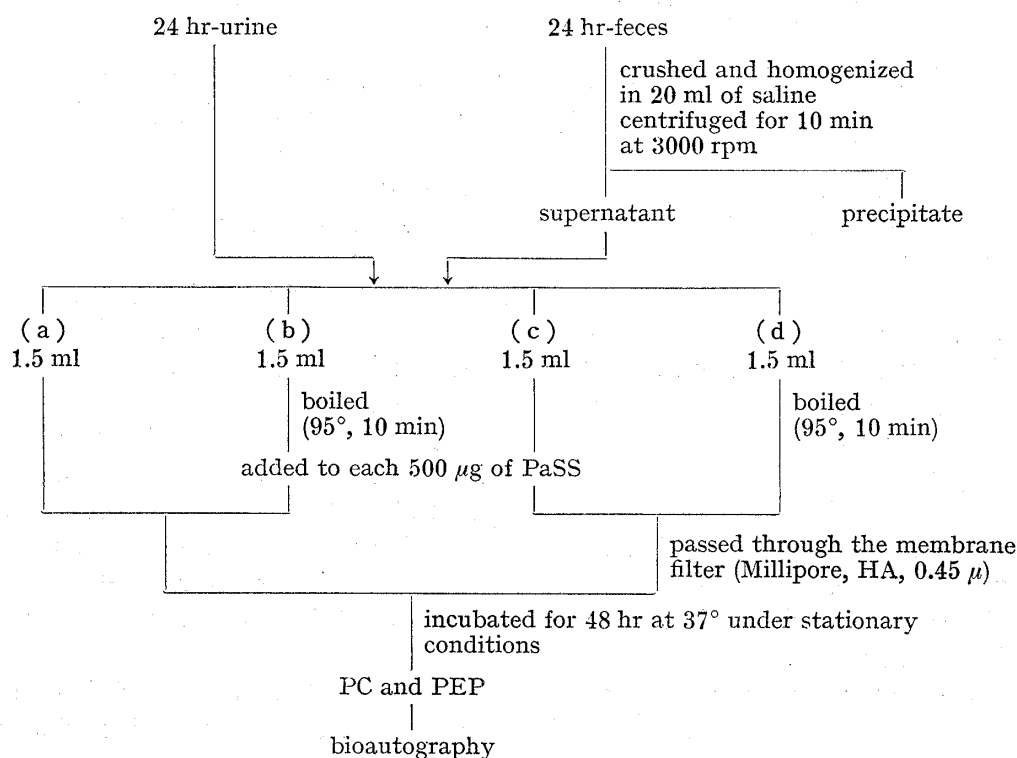


Chart 2. Procedures for Examination of The Formation of PaSSO_3H from PaSS in Urine and Feces of Rat

Aqueous solution of PaSS was passed through a column of QAE-Sephadex (OH^-) to remove trace amount of PaSSO_3H . Conditions of PC, PEP, and bioautography were same as in Experimental.

TABLE I. PaA Derivatives found in Specimens and Feed

Sample	PaA derivative
¹⁴ C-PaSS-administered rat ^{a)} :	
Urine	$\text{PaA} > \text{PaSS} > (\text{P-PaA} \text{ \&/or } \text{PaSSO}_3\text{H}) > \text{P-PaSSO}_3\text{H}$
Feces	$\text{PaA} > \text{PaSS} > \text{PaSSO}_3\text{H} > \text{P-PaA}$
Control rat ^{b)} :	
Urine	$\text{PaA} > \text{P-PaA}$
Feces	$\text{PaA} > \text{PaSS} > \text{P-PaA}$
Feed ^{b)}	PaA

a) Analyzed by radioactivity measurement supplemented by bioautography.

b) Analyzed by bioautography.

34) I.M. Kolthoff and W. Stricks, *J. Am. Chem. Soc.*, **73**, 1728 (1951); I. Kolthoff, W. Stricks, and R.C. Kapoor, *ibid.*, **77**, 4733 (1955); T.W. Szczepkowski, *Nature*, **182**, 934 (1958).