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Bifidus Factors in Carrot. II.[1] The Structure of the Factor in Fraction IV[2]

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Molecular weight of the major bifidus factor (I) in carrot was less than 700. I was strongly acidic and converted to a still strongly acidic active compound (II) with alkaline phosphatase. I and II changed to D-pantetheine 4'-phosphate (P-PaSH) and D-pantetheine (PaSH) with KCN or 2-mercaptoethanol, conversely in 0.1M $NaHCO_3$, P-PaSH and PaSS (D-pantethine) gradually and partially changed to I and II, respectively. These fact suggested that I and II were oxidation products of P-PaSH and PaSS. Among possible oxidation products of PaSS synthesized, D-Pantetheine-S-sulfonic acid ($PaSSO_3H$) was active and coincided with II. Synthesized 4'-phospho-D-pantetheine-S-sulfonic acid ($P\text{-}PaSSO_3H$) was also active and identified with I by the behaviors in thin-layer chromatography, paper electrophoresis, amino acid analysis, infrared and nuclear magnetic resonance spectra. The possibility to be an artifact of $P\text{-}PaSSO_3H$ was deniable, since a column chromatogram on DEAE-Sephadex of direct methanol extract of raw carrot root showed the identical peak. The biochemical and medical significances of the new compound $P\text{-}PaSSO_3H$ are discussed.

In the previous paper we reported purification and some properties of bifidus factors in carrot (*Daucus Carota* L.). Among five kinds of growth factors separated, the major growth factor (I) in fraction IV was highly purified (200000 times) using a common strain N4 of *Bifidobacterium bifidum* for growth activity determination.[1] In the present paper, the structure of I was investigated.

Result

At the first time, the molecular weight of I was measured by gel filtration on a column of Sephadex G-10. From the retention volume shown in Table I and its strongly acidic charactor, the molecular weight was suggested to be less than 700.

Table I. Gel Filtration on Sephadex G-10 in 0.1M $HCOONH_4$

Compound	M.W.[a]	Loaded dose	E.V.[b]	Detection
Blue dextran	2×10^6	0.3 mg	13 ml	absorbance at 650 mμ
Cyanocobalamine	1355	0.3 mg	13	absorbance at 361 mμ
Coenzyme A	768[c]	1 mg	13	absorbance at 259 mμ
P-PaSS	715[c]	2 mg	13	chemical reaction[4]
I		4000 units	15	bioassay
$P\text{-}PaSSO_3H$	469	1 mg	15	bioassay and chemical reaction[4]
PaSS	555	2 mg	16	chemical reaction[4]
K_2HPO_4	174	10 mg	16	chemical reaction[5,6]
Glucose	180	10 mg	18	chemical reaction[7]

a) molecular weight *b*) elution volume *c*) free form

1) Part I: K. Samejima, M. Yoshioka, and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **19**, 166 (1971).
2) A part of this work was presented at the 90th Annual Meeting of Pharmaceutical Society of Japan, Sapporo, July 1970.
3) Location: *Hongo, Bunkyo-ku, Tokyo, 113, Japan.*
4) G. Toennis and J.J. Kolb, *Anal. Chem.*, **23**, 823 (1951).
5) C.S. Hanes and F.A. Isherwood, *Nature*, **164**, 1107 (1949).
6) R.S. Bandurski, *J. Biol. Chem.*, **193**, 405 (1951).
7) "Dyeing reagents for thin layer and paper chromatography," E. Merck AG., Darmstadt, Germany, 1961.

Table II. Determination of Amino Acids and Phosphoric Aicd produced from I and P-PaSS

Component\Sample	42 μg of I[a)]	0.05 μmole of P-PaSS
β-Alanine	0.020 μmole	0.082 μmole
Taurine	0.019	0.079
Glutamic acid	0.008	0
Glycine	0.004	0
Phosphoric acid	0.110	0.076

a) The quantity corresponds to 0.02 μmole calculated from the microbiological activity comparing with the specific activity (400 units/mμmole) of P-PaSSO$_3$H.

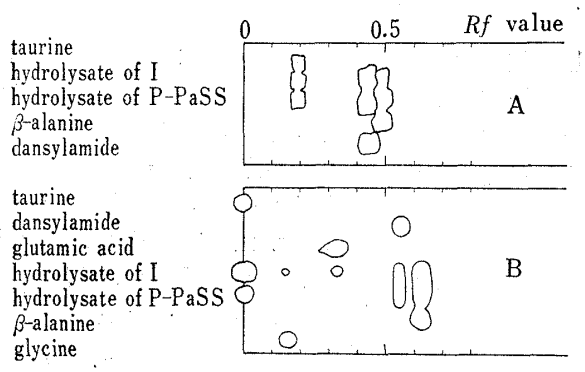

Fig. 1. Thin-Layer Chromatograms of Dansyl Derivatives of the Hydrolysates of I and P-PaSS on Nylon Sheets

A was developed with H_2O–90% HCOOH (100:1.5 v/v) and B with benzene–AcOH (9:1 v/v). The separated dansyl amino acids were visualized by ultraviolet irradiation (365 mμ).

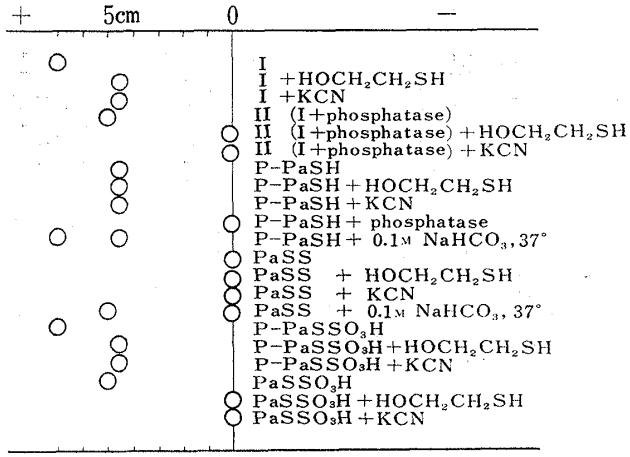

Fig. 2. Bioautogram of a Paper Electrophorogram on Toyo Roshi No. 51A

Filter paper in 1M HCOOH, pH 2 at 11.8 V/cm for 2 hours.

After performic acid oxidation and hydrochloric acid hydrolysis of I, equimolecular β-alanine and taurine was estimated with an amino acid analyzer, together with smaller amounts of glutamic acid and glycine which were probably derived from the paper used in electrophoresis.[1)] These amino acids were also detected by dansylation followed by thin-layer chromatography (Fig. 1). Furthermore, an unexpected large amount of phosphoric acid was detected from the sample probably due to contamination from the paper and/or with organic phosphates. These results are summarized in Table II.

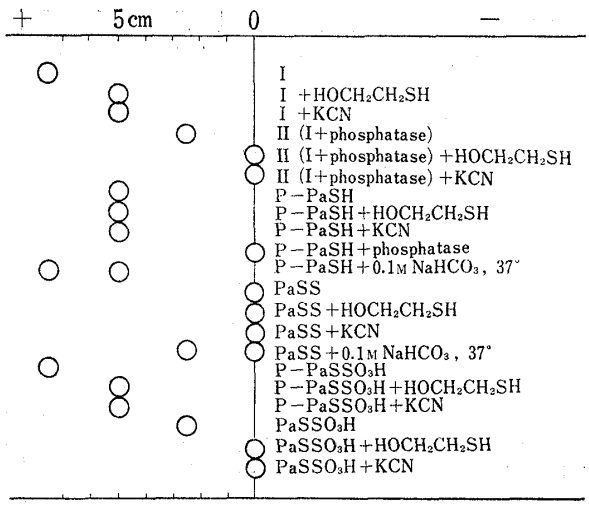

Fig. 3. Bioautogram of a Paper Electrophorogram on Toyo Roshi No. 51A

Filter paper in 0.1M NH_4HCO_3, pH 8 at 11.8 V/cm for 2 hours.

In paper electrophoresis at pH 2 or 8, I moved faster than P-PaSH against anode (Fig. 2,3). By dansylation, the activity and electrophoretic mobility of I did not alter, which suggested no existence of free amino group in it. With an alkaline phosphatase, another

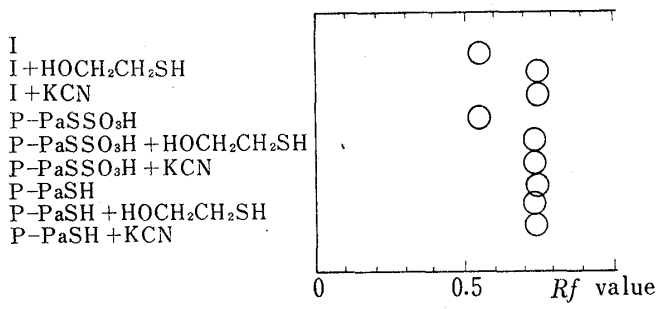

Fig. 4. Bioautogram of a Thin-Layer Chromatogram on Avicel SF developed with 2-Propanol–H_2O (3:1 v/v)

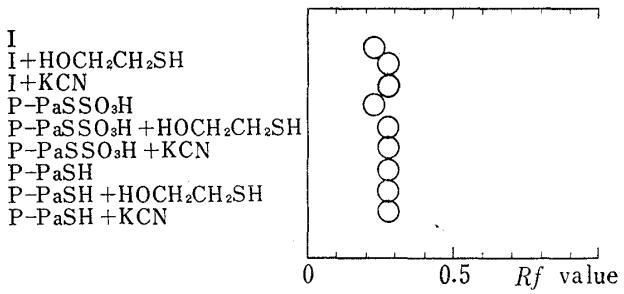

Fig. 5. Bioautogram of a Thin-Layer Chromatogram on Avicel SF developed with 1-Butanol–AcOH–H_2O (5:2:3 v/v)

strongly acidic active substance (II) was produced. At pH 8, the mobilities of I and P-PaSH were three and two times as large as II, respectively (Fig. 3).

With KCN or 2-mercaptoethanol, I and II were converted to P-PaSH and PaSH (Fig. 2—7). Conversely, P-PaSH and PaSS in 0.1M $NaHCO_3$ at 37° were gradually and partially converted to I and II respectively (Fig. 2, 3). Thus I and II were suggested as oxidation products of P-PaSH and PaSS.

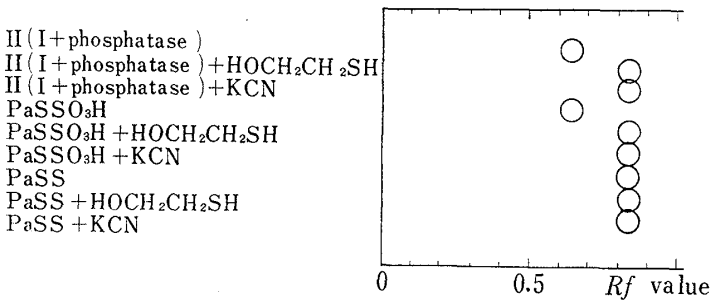

Fig. 6. Bioautogram of a Thin-Layer Chromatogram on Silica Gel H developed with 2-Propanol–H_2O (2:1 v/v)

Among three possible types of oxidation products, RSO_2H, RSO_3H and $RSSO_3H$, suggested from a previous work,[8] RSO_2H was denied since cysteine sulfinic acid was unstable and was not reduced with KCN as shown in Fig. 8, while I was stable during the long purification period and reducible with KCN and therefore the compounds of the remaining two types were synthesized for comparison as shown in Fig. 9.

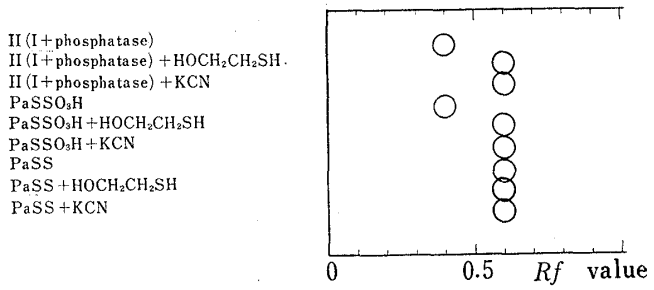

Fig. 7. Bioautogram of a Thin-Layer Chromatogram on Silica Gel H developed with 1-Butanol–AcOH–H_2O (5:2:3 v/v)

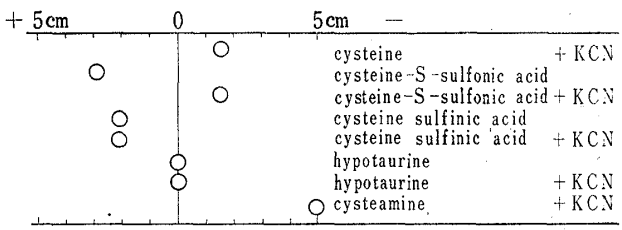

Fig. 8. Paper Electrophorogram on Toyo Roshi No. 51A Filter Paper in 1M HCOOH, pH 2 at 11.8 V/cm for 2 Hours

The spots were detected with the ninhydrin reagent.[8]

Even 10 μmoles of $PaSO_3H$ was inactive. However, $PaSSO_3H$ showed high activity and coincided with II in the behaviors in thin-layer chromatography and paper electrophoresis (Fig. 2—7). Synthesized P-$PaSSO_3H$ (Fig. 9) was also powerfully active and identical with

8) W.E. Savige and J.A. Maclaren, "The chemistry of organic sulfur compounds," Vol. 2, ed. by N. Kharasch and C.Y. Meyers, Pergamon Press Inc. N. Y., p. 1966, p. 367.
9) A.R. Fahmy, A. Niederwieser, G. Pataki, and M. Brenner, *Helv. Chim. Acta*, **44**, 2022 (1961).

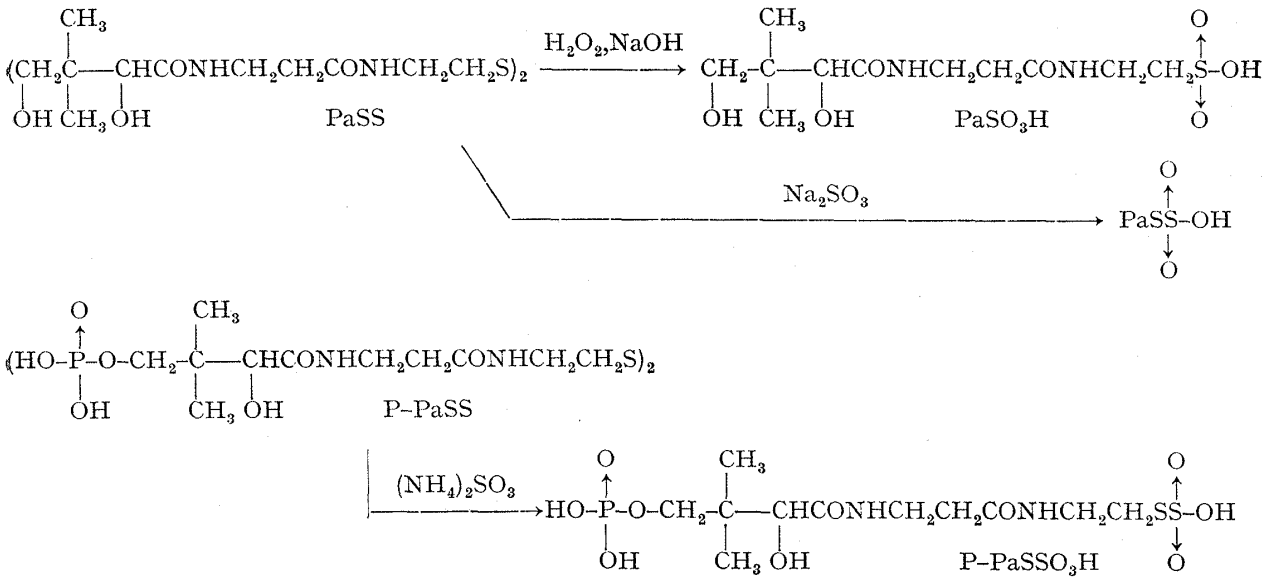

Fig. 9. Synthesis of $PaSO_3H$, $PaSSO_3H$ and P-$PaSSO_3H$

I in the above characteristics and in the infrared (IR) and nuclear magnetic resonance (NMR) spectrum neglecting some comtaminated peaks (Fig. 2—7, 10, 11). P-$PaSSO_3H$, $PaSSO_3H$ and P-PaSH and 1/2 mole of PaSS showed the same activity (400 units per 1 mμmole) and similar growth pattern as I (Fig. 12) suggesting that they will act as bifidus factors through a common form. Compared with P-$PaSSO_3H$ by the microbiological activity and the amino acid contents, I was about 25% pure.

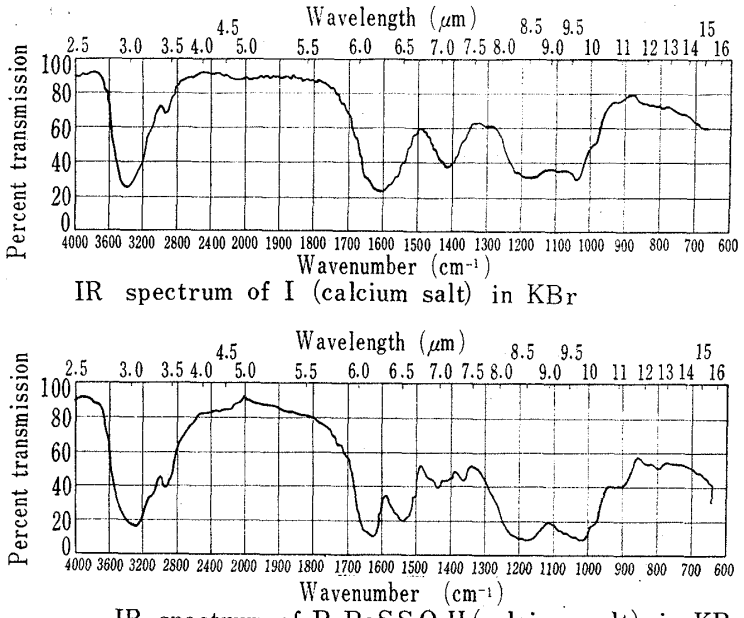

Fig. 10. IR Spectra of I and P-$PaSSO_3H$

There remained a possibility that P-$PaSSO_3H$ might be artificially produced in the first powdering step of carrot root, while there was no drastic reaction in the following steps and any production of P-$PaSSO_3H$ was not observed in DEAE-Sephadex (OH) column chromatography of P-PaSH.[1] So, we extracted a raw carrot root with methanol and the activities in the methanol extract was separated on a column of QAE-Sephadex (OH). The active substance in the fifth peak (E) was identical with P-$PaSSO_3H$ as shown in Fig. 13 and the activity in the peak was also coincided with P-$PaSSO_3H$ by paper electrophoresis in the same manner described. Further the active substance in the second (B) peak was estimated as $PaSSO_3H$ by the same column chromatography and paper electrophoresis.

Discussion

In conclusion I is identified with P-$PaSSO_3H$ and its chemical behaviors described in the results are illustrated in Fig. 14.

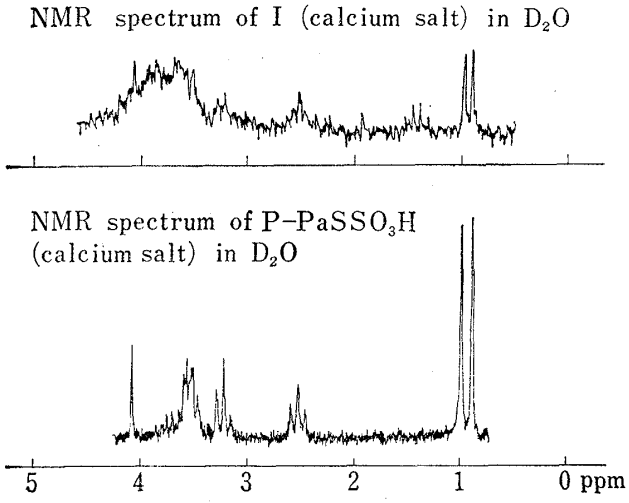

Fig. 11. NMR Spectra of I and P-PaSSO$_3$H

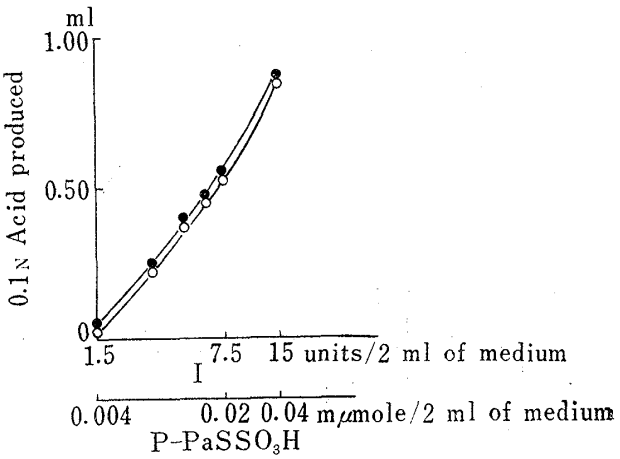

Fig. 12. Responses of *B. bifidum* H4 to I (-○-) and P-PaSSO$_3$H (-●-)

The discovery of the new compound, P-PaSSO$_3$H, from carrot is interesting in two points. Firstly, S-sulfonic acids have seldom been isolated from natural sources, probably because many SH-compounds isolated were usually treated with reductants such as 2-mercaptoethanol or dithiothreitol during extraction. Nakamura, *et al.* found that *Aspergillus nidulans* synthesized cysteine-S-sulfonic acid from sulfate and serine,[10] and Waley also found an existence of glutathione-S-sulfonic acid in calf eyes.[11] These and our findings suggest wide distribution of such compounds in nature. We are investigating the biological significance of P-PaSSO$_3$H. It may be possibly converted to CoA[12] or acyl carrier protein[13] through P-PaSH by *B. bifidum*.

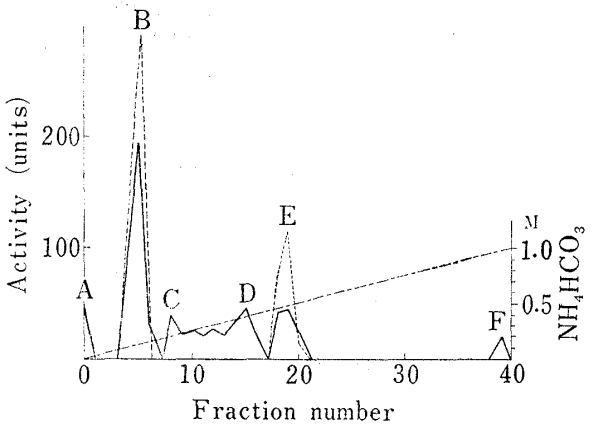

Fig. 13. QAE-Sephadex (OH) Column Chromatography of the Direct Methanol Extract of Carrot

———; the extract
----------; the extract plus P-PaSSO$_3$H
– – – –; the extract plus PaSSO$_3$H

Secondly, as P-PaSSO$_3$H is fairly stable and strongly acidic compound, it may reach to large intestines differently from PaSH, P-PaSH, PaSS, *etc.* to propagate *B. bifidum* and consequently it may represent the medical role of carrot. Since 1 g of the carrot powder (550 units/g) contains less than 0.68 μg of calcium salt of P-PaSSO$_3$H (810 units/μg), and Ohta, *et al.* stated that a soup of about 6 g of the carrot powder per day propagated *B. bifidum* in intestines of infants,[14] administration of 4 μg of P-PaSSO$_3$H per day would be sufficient.

Experimental

All evaporations were carried out *in vacuo* at 40°. The growth activity (unit) for *Bifidobacterium bifidum* N4 was determined by the method described in the previous paper.[1] NMR spectrum was measured with a

10) T. Nakamura and R. Sato, *Biochem. J.*, **86**, 328 (1963).
11) S.G. Waley, *Biochem. J.*, **71**, 132 (1959).
12) G.M. Brown, *J. Biol. Chem.*, **234**, 370 (1959).
13) P.W. Majerus, A.W. Alberts, and P.R. Vagelos, *Proc. Natl. Acad. Sci.*, **51**, 1231 (1964).
14) K. Ohta, Transaction of the 15th General Assembly of the Japan Medical Congress, Vol. 5, 1959, p. 232.

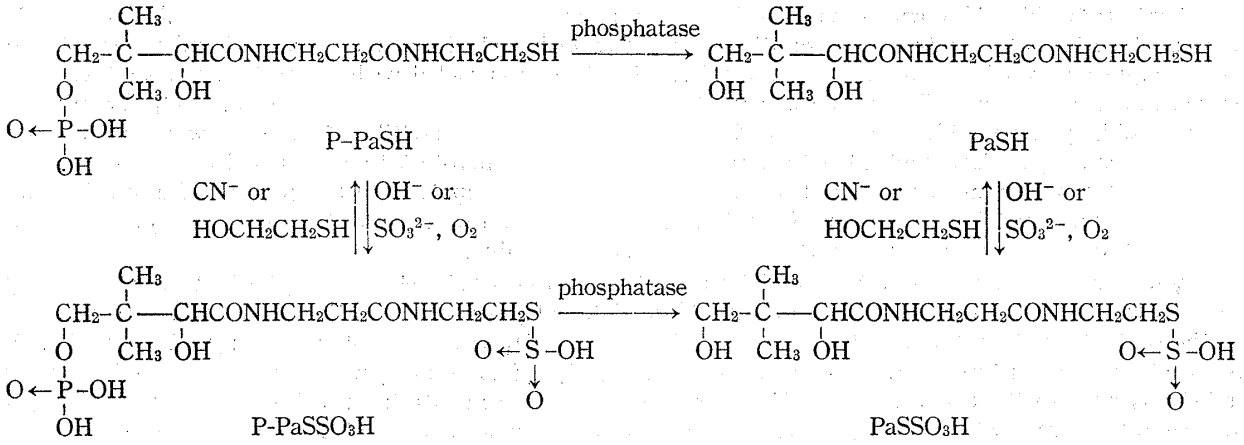

Fig. 14. Reaction of P-PaSSO$_3$H

Varian HR-100 Analytical Nuclear Magnetic Resonance Spectrometer in D_2O added with sodium 2,2-dimethyl-2-silapentane sulfonate as a internal standard. IR spectrum was measured with a Infrared Spectrometer Model DS-402G (Japan Spectroscopic Co., Ltd.) in KBr tablets.

Gel Filtration with Sephadex G-10——A column of Sephadex G-10 (1.5×20.5 cm) was bufferized with 0.1M $HCOONH_4$ (adjusted pH to 3 with HCOOH). The sample was dissolved in the same buffer, poured onto the column and eluted with the buffer (Table I).

Analysis of Amino Acid and Phosphoric Acid——I (70 μg) or calcium D-pantethine 4′,4″-diphosphate·$4H_2O$ (P-PaSS)[15] (603 μg) was dissolved in 100 μl of HCOOH (90%) at 0°. The solution was added with 100 μl of the mixture of HCOOH (90%) and H_2O_2 (30%) (9.5: 0.5 v/v) wihch was previously allowed to stand at room temperature for 3 hours and cooled to 0°, and kept for 4 hours and evaporated to dryness. The residue was dissolved in 0.5 ml of 6N HCl and sealed *in vacuo*, heated at 108° for a day and evaporated to dryness under a stream of N_2. The residue from I was dissolved in 0.5 ml of water, 300, 100 and 100 μl of which were applied to amino acid and phosphoric acid analysis and dansylation respectively. The residue from P-PaSS was dissolved in 1.5 ml of water and 100, 10 and 10 μl were similarly treated.

The amino acid analysis was carried out with an amino acid analyzer (Yanagimoto Model LC2). Dansylation (1-dimethylaminonaphthalene 5-sulfonylation) was done according to Gray, *et al.* (Fig. 1).[16] Phosphoric acid was determined according to Imai, *et al.* (Table II).[17]

Electrophoresis——I (40 units), P-PaSH and PaSS (0.25 μg each) were spotted on Toyo Roshi No. 51A. The paper was moistened spraying 1M HCOOH or 0.1M NH_4HCO_3, and applied horizontally to an appatus of Toyo Roshi Co. type C. Electrophoresis was done at 11.8 v/cm for 2 hours. The paper was dried and used for the bioautography according to Nakamura, *et al.*[18] I was also dansylated in the same manner described and applied to the paper electrophoresis (Fig. 2, 3).

Degradation with an Alkaline Phosphatase——Alkaline phosphatase (EC 3·1·3·1, 32 units/mg, C.F. Boehringer and Soehne GmbH, Germany) of 4 mg was dissolved in 10 ml of water in a cellophane tube and dialyzed against 1 liter of water at 4° for 2 days.

The enzyme solution was evaporated to dryness. The residue was dissolved in 0.4 ml of water. I and calcium D-pantetheine 4′-phosphate·$5H_2O$ (P-PaSH)[15] of 100 μg each were dissolved in 0.5 ml of 0.1M NH_4HCO_3 and added with 0.1 ml of the above enzyme solution. The mixture was incubated at 37.5° for a day and poured onto a coulumn of Sephadex G-25 bufferized with 0.1M $HCOONH_4$ of pH 3 and eluted with the same buffer. Two μl of 1 ml fraction was spotted on a filter paper and activity was detected by bioautography. The active fractions (No. 24 from I and No. 23 from P-PaSH) were applied to electrophosreis (Fig. 2, 3).

Reduction with KCN——I and II of 40 units each, P-PaSSO$_3$H (calcium 4′-phospho-D-pantetheine-S-sulfonate), PaSSO$_3$H (calcium D-pantetheine-S-sulfonate), P-PaSH, and PaSS of 0.25 μg each were spotted on a filter paper or a thin-layer. On the same places 20% w/v KCN was spotted three times (Fig. 2—7). Cysteine-S-sulfonic acid,[19] cysteine sulfinic acid,[19] hypotaurine,[19] cysteine, and cysteamine-HCl of 10 μg each were spotted on the filter paper. On the same places 50% w/v KCN was spotted three times. The paper was applied to electrophoresis (Fig. 8).

15) Kindly synthesized and provided by Daiichi Seiyaku Co., Ltd; O. Nagase, *Chem. Pharm. Bull.* (Tokyo), **15**, 648 (1967); M. Shimizu, G. Ohta, O. Nagase, and S. Okada, *ibid.*, **13**, 180 (1965).
16) W.R. Gray and B.S. Hartley, *Biochem. J.*, **89**, 59 (1963).
17) K. Imai and S. Akamatsu, *J. Biochem.*, **39**, 203 (1952).
18) H. Nakamura, K. Samejima, and Z. Tamura, *Japan J. Microbiol.*, **14**, 9 (1970).
19) Kindly provided by Mr. Isao Matsunaga, Research Laboratory, Chugai Pharmaceutical Co., Ltd.

Treatments with 2-Mercaptoethanol——The same quantity of the sample as used in reduction with KCN was dissolved in 1 μl of water. The solution was added with 1 μl of 2-mercaptoethanol and absorbed into a glass cappilary, which was putted in a test tube. The tube was sealed with a Parafilm and kept at room temperature for 2 hours. The sample was spotted on a filter paper or a thin-layer (Fig. 2—7). Cysteine-S-sulfonic acid, cysteine sulfinic acid, hypotaurine, cysteine, cysteamine-HCl of 1 mg each were dissolved in 0.1 ml of 2-mercaptoethanol added with a drop of water. The microtest tube was sealed with a Parafilm and kept at room temperature for 35 hours. The sample was spotted on the paper and applied to the electrophoresis. Cysteine-S-sulfonic acid and cysteine sulfinic acid were converted to cysteine judging from their mobilities. Hypotaurine was also converted to cysteamine.

Synthesis of Calcium D-Pantothenoyl Taurine (PaSO$_3$H)——PaSO$_3$H was synthesized from PaSS by Stewart, *et al.*[20] But the yield was low (8.2%) and detailed physical properties were not described. We synthesized PaSO$_3$H neutralizing the solution of PaSS and H_2O_2 with drops of NaOH in good yield (Fig. 9). D-Pantethine[15] of 555 mg (1 mmole) and 0.6 ml of 30% H_2O_2 (5.9 mmoles) were dissolved in 4 ml of water and slowly added with drops of 1N NaOH. The excess NaOH was neutralized with dry ice and excess H_2O_2 was degradated with 20 mg of 5% paradium-carbon in a refrigerator overnight. The charcoal was filtered off. The solution was poured onto a column of DEAE-Sephadex (OH, 1.5×28 cm). The column was washed with 100 ml of water and eluted with a linear gradient of ammonium bicarbonate. There was 500 ml of water in the mixing flask and 500 ml of 0.25M NH_4HCO_3 in the reservior. 50 ml fractions were collected and evaporated to dryness. The residue in No. 4—5 was passed through 30 ml of SE-Sephadex (Ca). The calcium salt was dissolved in a small volume of methanol. Insoluble $CaCO_3$ was centrifuged off and the supernatant was evaporated to dryness. The centrifugation step was repeated two times. The residue was dissolved in water and evaporated to dryness. A humid white powder was dried *in vacuo* at 60° for 2 hours. Yield, 500 mg (73%). mp 171° (softening, corr.). *Anal.* Calcd. for $C_{11}H_{21}O_7N_2SCa_{0.5}$: C, 38.25; H, 6.13; N, 8.11; S, 7.60. Found: C, 38.27; H, 6.51; N, 7.57; S, 7.84. $[\alpha]_D^{20}=+26°$ (c=2.22, H_2O). IR ν_{max}^{KBr} cm^{-1}: 3320, 3110, 2970, 2880, 1655, 1638, 1562, 1545, 1490—1405, 1400—1360, 1250, 1230—1185, 1180—1150, 1080, 1043, 988, 740.

Synthesis of Calcium D-Pantetheine-S-sulfonate (PaSSO$_3$H)——PaSSO$_3$H was synthesized by reaction of PaSS with Na_2SO_3, according to the synthesis of cysteine-S-sulfonic acid by Koltoff, *et al.* (Fig. 9).[21] D-Pantethine[15] of 555 mg (1 mmole) was dissolved in 100 ml of 0.2M each solution of Na_2SO_3 and NH_4OH. The solution was bubbled with air through 0.2M NH_4OH at room temperature for 2 hours, and further bubbled with air through water for 20 hours. The solution was evaporated to dryness and the residue was extracted with methanol. The methanol solution was evaporated to dryness. The residue was dissolved in water and poured onto a column of QAE-Sephadex (CO_3) (1.5×19 cm). The column was washed with 100 ml of water and eluted with a linear gradient of ammonium bicarbonate. There was 500 ml of water in the mixing flask and 500 ml of 0.5M NH_4HCO_3 in the reservoir. 50 ml fractions were collected and evaporated to dryness. The residue in No. 5—6 fractions positive to nitroprusside-KCN reaction[4] was changed to calcium salt through a column of 40 ml of SE-Sephadex (Ca). The salt was dissolved in a small volume of methanol, insoluble compounds were centrifuged off and the supernatant was evaporated to dryness. The centrifugation step was repeated two times. The residue was dissolved in water and evaporated to dryness. A humid white powder was obtained. Yield, 213 mg (28%). mp 174° (softening, corr.). *Anal.* Calcd. for $C_{11}H_{21}O_7N_2S_2Ca_{0.5}$: C, 34.81; H, 5.59; N, 7.38; S, 16.87. Found: C, 34.85; H, 5.73; N, 7.09; S, 16.54. $[\alpha]_D^{22°}=+13.9°$ (c=1.94, H_2O). IR ν_{max}^{KBr} cm^{-1}: 3320, 3100, 2950, 2880, 1650, 1635, 1560, 1548, 1480—1410, 1390—1360, 1290, 1250—1165, 1082, 1015, 985.

Synthesis of Calcium 4′-Phospho-D-pantetheine-S-sulfonate (P-PaSSO$_3$H)——Calcium D-pantetheine 4′,4″-diphosphate·4H_2O[15] of 431 mg (0.5 mmole) was dissolved in 100 ml of 0.2M $(NH_4)_2SO_3$ and NH_4OH. The solution was bubbled with air through 0.2M NH_4OH at room temperature for 2 hours, and further bubbled with air through water for 20 hours. The precipitate of $CaCO_3$ was removed by decantation. The supernatant was evaporated to dryness. The residue was dissolved in water and poured onto a column of QAE-Sephadex (OH) (1.5×19 cm). The column was washed with 100 ml of water and eluted with a linear gradient of ammonium bicarbonate. There was 500 ml of water in the mixing flask and 500 ml of 0.5M NH_4HCO_3 in the reservoir. 50 ml farctions were collected and evaporated to dryness. The residue in No. 9—10 positive to nitroprusside-KCN reaction[4] was changed to calcium salt through 40 ml of SE-Sephadex (Ca). The salt was dissolved in a little water, and insoluble compounds were centrifuged off. The supernatant was evaporated to dryness. The centrifugation step was repeated two times. A humid white powder was obtained. Yield, 283 mg (57%). *Anal.* Calcd. for $C_{11}H_{20}O_{10}N_2S_2Ca_{1.5}P$: C, 26.66; H, 4.08; N, 5.65; S, 12.94; P, 6.25. Found: C, 26.61; H, 4.56; N, 5.53; S, 13.37; P, 6.18. $[\alpha]_D^{22°}=+7.2°$ (c=1.95, H_2O). IR and NMR spectra were shown in Fig. 10, 11.

Methanol Extraction of Carrot——Carrot (*Daucus carota* L.) roots (100 g) were homogenized in methanol for a minute with a mixer and allowed to stand at room temperature for an hour. Insoluble materials

20) C.J. Stewart, V.H. Cheldelin, and T.E. King, *J. Biol. Chem.*, **215**, 319 (1955).
21) I.M. Koltoff and W. Stricks, *J. Am. Chem. Soc.*, **73**, 1728 (1951).

were filtered off and the solution was evaporated to dryness (5.25 g, 3.5 units/mg). Furthermore, when P-PaSH (4.9×10^4 units) was added to methanol and homogenized with the roots (100 g) in the same manner, 100% recovery of P-PaSH and no production of P-$PaSSO_3H$ were confirmed by the following QAE–Sephadex column chromatography and paper electrophoresis.

QAE-Sephadex (OH) Column Chromatography of the Methanol Extract——The extract of 300 mg (or plus P-$PaSSO_3H$ of 200 units or $PaSSO_3H$ of 200 units) was dissolved in a small volume of water and poured onto a column (0.9×11 cm) of QAE–Sephadex (OH). The column was washed with water and eluted with a linear gradient of ammonium bicarbonate (0—1M) (total 100 ml). Fractions of 5 ml each were collected, evaporated to dryness, and assayed. The total activity recovered was 1043 units (100%). If added, the activity of P-$PaSSO_3H$ or $PaSSO_3H$ was almost recovered (Fig. 13). Fraction B and E corresponding to 40 units were spotted on a paper, applied to paper electrophoresis and showed the same mobilities as $PaSSO_3H$ and P-$PaSSO_3H$ respectively.

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