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The Production of Bisulfite from Pantetheine-S-sulfonic Acid by *Bifidobacterium bifidum*[1]

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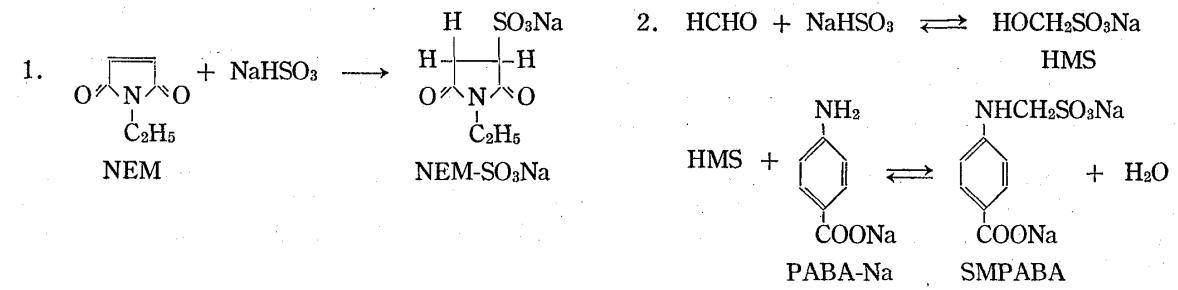
Identification of the radioactive metabolite of $PaS^{35}SO_3H$ in *B. bifidum* N4 was undertaken. The strain was incubated with $(PaS^{35}SO_3)_2Ca$ for 60 min at 37° in CySS (−) MGM, and after removal of the bacteria, the medium which showed a new radioactive peak on a chromatogram was neutralized and reacted with NEM or HCHO and PABA. By this treatment, the peak disappeared and in turn a new peak emerged in both cases. They were identified as NEM-$^{35}SO_3H$ and SMPABA-^{35}S by PEP (1 M HCOOH, pH 2) and radiochemical crystallizations.

In the absence of the bacteria, the peak of the radioactive metabolite was not produced by the incubation of $(PaS^{35}SO_3)_2Ca$ with CySS(−)MGM, neither produced NEM-$^{35}SO_3H$ from $(PaS^{35}SO_3)_2Ca$ by NEM treatment. Furthermore, transformation of $^{35}SO_4^{2-}$ to $H^{35}SO_3^-$ by the strain was not observed. These results demonstrate the production of HSO_3^- from $PaSSO_3H$ by *B. bifidum* N4.

In a previous communication,[3] it was reported that radioactive $PaS^{35}SO_3H$ was taken up by *Bifidobacterium bifidum* N4 by a physiological active transport followed by intracellular degradation to PaSH and $^{35}SO_4^{2-}$. However, the identification of $^{35}SO_4^{2-}$ was uncertain, because the procedure employed was aerobic to the extent that $^{35}SO_3^{2-}$, if present, would be oxidized to $^{35}SO_4^{2-}$. In the present investigation, therefore, reexamination of the metabolite with ^{35}S activity from $PaS^{35}SO_3H$ was undertaken by using the following two reaction systems for HSO_3^- in which SO_4^{2-} does not participate:



1) This work was presented at the 94th Annual Meeting of Pharmaceutical Society of Japan, Sendai, April, 1974. The abbreviations used in this paper are: PaSH, pantetheine; PaSS, pantethine; $PaSSO_3H$, pantetheine-S-sulfonic acid; $(PaSSO_3)_2Ca$, calcium salt of $PaSSO_3H$; $CySSO_3H$, L-cysteine-S-sulfonic acid; NEM, N-ethylmaleimide; NEM-SO_3H, N-ethylsuccinimide-3-sulfonic acid; NEM-SO_3Na, sodium salt of NEM-SO_3H; HMS, hydroxymethanesulfonic acid sodium salt; PABA, *p*-animobenzoic acid; PABA-Na, sodium salt of PABA; SMPABA, 4-(sulfomethyl)amino-benzoic acid disodium salt; MeOH, methyl alcohol; EtOH, ethyl alcohol; PC, paper chromatography; PEP, paper electrophoresis; CySS-(−)MGM, the basal medium[4] omitted with L-cystine.
2) Location: *Hongo-7-3-1, Bunkyo-ku, Tokyo.*
3) H. Nakamura and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **19**, 1516 (1971).
4) M. Yoshioka, S. Yoshioka, Z. Tamura, and K. Ohta, *Japan. J. Microbiol.*, **12**, 395 (1968).

Experimental[5]

1. Chemicals and Materials——Sodium sulfite-^{35}S ($Na_2{}^{35}SO_3$, specific activity, 2.3 mCi/mmole) was purchased from The Radiochemical Center (England). Sodium sulfate-^{35}S ($Na_2{}^{35}SO_4$, specific activity, 11.7 mCi/mmole) was obtained from Daiichi Pure Chemicals Co., Ltd., Tokyo. Pantethine (PaSS, 57.2% aqueous solution) and pantetheine-S-sulfonic acid calcium salt monohydrate ($(PaSSO_3)_2Ca$) were kindly provided by Daiichi Seiyaku Co., Ltd., Tokyo. N-Ethylmaleimide (NEM, specially purified reagent) was purchased from Nakarai Chemicals, Ltd., Kyoto. *p*-Aminobenzoic acid (PABA, GR) and hydroxymethanesulfonic acid sodium salt ($HOCH_2SO_3Na \cdot H_2O$, HMS, GR) were purchased from Tokyo Kasei Kogyo Co., Ltd., Tokyo. Formalin (HCHO, GR, assay minimum 37.0%), sodium bisulfite ($NaHSO_3$, GR) and sodium sulfite (Na_2SO_3, GR) were obtained from Kanto Chemical Co., Inc., Tokyo. QAE-Sephadex A-25 was the product of Rohm and Haas Co., USA. Wakogel FM plates (5×10 cm) were purchased from Wako Pure Chemical Industries, Tokyo. Other chemicals and solvents were all guaranteed grade.

2. Microorganism and Culture——*Bifidobacterium bifidum* N4 was used throughout this investigation. The preculture medium was identical with that described by Yoshioka, *et al.*,[4] and the basal medium used which designated as CySS (−) MGM in this paper was prepared from that of Yoshioka, *et al.*[4] by omitting L-cystine. All the cultures of the strain were performed in an atmosphere of N_2-CO_2 (9: 1, v/v) at 37°.

3. Bioautographic Detection of Pantetheine Derivatives——PaSS and $PaSSO_3H$ were detected by bioautography[6] with *B. bifidum* N4 as an indicator microorganism on the basal medium[4] supplemented with 1.5% Bacto-agar.

4. Synthesis of Pantetheine-S-Sulfonic Acid Calcium Salt-^{35}S ($(PaS^{35}SO_3)_2Ca$)——PaSS (55.4 mg, 0.1 mmol) and $Na_2{}^{35}SO_3$ (39.4 mg, 0.3 mmole) were dissolved in 50 ml of 0.1 M NH_4OH and aerated for 18 hr at room temperature by air passed through 200 ml of 0.1 M NH_4OH. The reaction mixture was evaporated to dryness below at 40° *in vacuo* and extracted four times with each 10 ml of MeOH.[7] After evaporation of the combined extracts to dryness, the resulting syrupy compounds were dissolved in 1 ml of water[8] and fractionated on a column of QAE-Sephadex A-25 (OH form, 0.9×11 cm) with a linear gradient of NH_4HCO_3 (0—0.5 M, 300 ml). Each 5 ml portions were collected under a constant rate of flow (32 ml/hr). Aliquots (5 μl) of the fractions were dried in planchets under a lamp and measured for radioactivity with a low background gas flow counter (Aloka, type SC-5). Although the chromatogram showed almost one peak, the radioactive peak (frs. 6 to 10) proved to be a mixture of $PaS^{35}SO_3H$, $^{35}SO_3{}^{2-}$ and/or $^{35}SO_4{}^{2-}$ by PC (*n*-BuOH: AcOH: H_2O=5: 2: 3, v/v) and PEP (0.1 M NH_4HCO_3, pH 8), therefore, $PaS^{35}SO_3H$ was purified by preparative PC as follows. The radioactive fractions were pooled and evaporated to dryness. A large amount of NH_4HCO_3 was removed by repeating evaporation with water. The residue was dissolved in a minimum volume of water, applied onto a Toyo Filter Paper No. 514 in a line and developed with the solvent system described above. The radiochromatogram of the developed paper showed a main peak at *Rf* 0.38 and also broad complicated peaks between at *Rf* 0 to 0.2. In comparison of *Rf* values of them with those of the co-chromatographed authentic compounds, it was supposed that the former was $PaS^{35}SO_3H$ and the latter was a mixture of inorganic oxides of ^{35}S. After air-drying the paper chromatogram, the area corresponding to the peak with *Rf* 0.38 was extracted with 100 ml of water and condensed to 2 to 3 ml. The condensate was charged onto a column of Amberlite CG-120 (Ca form, 1.2×10 cm), which was prepared from the commercial resin (Na form) by washing with 2 N HCl, water, saturated $CaCl_2$ and water in this order, and eluted with 100 ml of water. The eluate was evaporated to dryness and dried over P_2O_5. Isotopically and bioautographically pure white residue of $(PaS^{35}SO_3)_2$ Ca was obtained (8.4 mg, yield 10.6%).

5. Detection of UV-Absorbing Compounds——The ultraviolet (UV)-absorbing compounds such as NEM, NEM-SO_3H, PABA and SMPABA were detected on papers or Wakogel FM plates by the mixed fluorescent materials method in a manner previously described.[9]

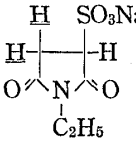
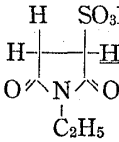
6. Synthesis of N-Ethylsuccinimide-3-Sulfonic Acid Sodium Salt (NEM-SO_3Na)——To $NaHSO_3$ (4.16 g, 0.04 mole) in 50 ml of water was dropwisely added NEM (5.01 g, 0.04 mole) in 50 ml of EtOH and stirred for 2 hr at 30°. The reaction mixture was condensed and was added with EtOH. The resulting precipitate was recrystallized from H_2O–EtOH and washed with ether to give NEM–SO_3Na, mp 252—254° (decomp.), as white needles in 97% yield (9.24 g); *Rf* 0.64, single red spot under UV rays on Wakogel FM plate developed with *n*-BuOH: AcOH: H_2O=5: 2: 3, v/v. *Anal.* Calcd. for $C_6H_8O_5NSNa \cdot 1/2H_2O$: C, 30.26; H, 3.81; N, 5.88. Found: C, 30.46; H, 3.41; N, 5.98. IR ν_{max}^{KBr} cm^{-1}: 692, 798, 808, 946, 1058, 1136, 1215, 1246, 1345,

5) All melting points are uncorrected. Nuclear magnetic resonance (NMR) spectra were recorded with JEOL NMR Spectrometer Model JNM 3H-60 at 60 MHz using 3-(trimethylsilyl)-propanesulfonic acid sodium salt (DSS) as an internal standard. Infrared (IR) spectra were recorded with JASCO Infrared Spectrophotometer Model DS-402 G.

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

7) All the evaporation were performed with a rotary evaporator below at 40° *in vacuo* unless otherwise indicated.

8) The glass-distilled water was used throughout this work.

1414, 1450, 1692, 1768, 2980, 3440. NMR (D_2O)τ: 8.90 (3H, t, $-CH_2C\underline{H}_3$), 6.91 (2H, q, $-C\underline{H}_2CH_3$), 6.45 (2H, d, ), 5.70 (1H, t, ).

7. Synthesis of 4-(Sulfomethyl)Amino-Benzoic Acid Disodium Salt (SMPABA)——PABA (13.7 g, 0.1 mole) was dissolved in 50 ml of 8% NaOH and adjusted to pH 7.2 with 2 N NaOH. To the resulting orange-yellow solution $HOCH_2SO_3Na \cdot H_2O$ (15.2 g, 0.1 mole) was added and stirred for 3 hr at 30°. The reaction mixture was added with EtOH and the precipitate was recrystallized from H_2O–EtOH followed by washing with ether to give SMPABA, mp 76—79° (decomp.) as white needles in 96% yield (28.1 g); *Rf* 0.34, single blue spot under UV rays on Wakogel FM plate developed with *n*-BuOH: AcOH: H_2O (5: 2: 3, v/v). *Anal.* Calcd. for $C_8H_7O_5NSNa_2 \cdot H_2O$: C, 32.77; H, 3.07; N, 4.78. Found: C, 32.63; H, 3.03; N, 5.03. IR ν_{max}^{KBr} cm^{-1}: 785, 1053, 1175, 1190, 1223, 1237, 1406, 1548, 1598, 1614, 3350. NMR (D_2O) τ: 5.50 (2H, s, $-NH-C\underline{H}_2-SO_3Na$), 3.12 (2H, d, aromatic hydrogens), 2.21 (2H, d, aromatic hydrogens).

8. Analysis of Metabolites——a) QAE-Sephadex Column Chromatography: The sample was charged onto a column of QAE-Sephadex A-25 (OH form, 0.8×12 cm) and a linear gradient elution was performed with 300 ml of 0—0.5 M NH_4HCO_3. The flow rate was 32 ml/hr. An aliquot (0.5 ml) of the collected fraction (5 ml) was measured for radioactivity in 10 ml of Bray's solution[10] with Packard Scintillations Spectrometer Model 3214.

b) Paper Electrophoresis: PEP was performed on Toyo Filter Paper No. 514 in 1 M HCOOH (pH 2) for 2 hr at 11.8 v/cm. The apparatus used was SJ-1050 (A) (Mitsumi Scientific Ind., Co., Ltd., Tokyo). Radioactive compounds were detected with Packard Radiochromatogram Scanner Model 7200 using the following conditions: time constant, 100 sec; linear scale, 3×10^2 cpm; chart speed, 0.1 cm/min; flow rate of He, 300 ml/min. Detection of PaSS and PABA were performed by bioautography and the mixed fluorescent materials method respectively.

c) Radiochemical Recrystallization: The radioactive derivative of the metabolite from $PaS^{35}SO_3H$ was extracted three times with each 5 ml of water from the air-dried paper electrophoretogram and the combined extracts were evaporated to dryness after addition of either authentic NEM-$SO_3Na \cdot 1/2H_2O$ (25 mg) or SMPABA·H_2O (22 mg). The residue was dissolved in a minimum volume of water and recrystallized by adding an appropriate solvent to constant specific activity. The radioactivity of the crystalline was measured in 20 ml of Bray's solution[10] with the instrument described in the preceding section a).

Result

Identification of Bisulfite by NEM Treatment

B. bifidum N4 grown overnight in 12 ml of the preculture medium[4] was washed three times with 0.9% NaCl, suspended in 2 ml of the same solution and incubated at 37° for 60 min after addition of 2 ml of CySS (—) MGM containing $(PaS^{35}SO_3)_2Ca$ (2.92×10^5 cpm). The culture was centrifuged for 5 min at 4000 rpm and the supernatant was filtered through a membrane filter (Millipore, type HA, pore size 0.45 μ, 25 mm i.d.). Four ml of the filtrate was divided into two and the one was added with 1 ml of 0.2 M NEM in 0.1 M phosphate buffer (pH 7.0) and the other was added with 1 ml of water as a control, and then the both were incubated for 30 min at 37° with constant shaking (150 oscillation/min) after adjusting the pH to 7 with 0.1 M K_2HPO_4.

Figure 1 shows the elution patterns of the filtrates on the QAE-Sephadex A-25 column. Two peaks, major one (81%) corresponding to authentic $PaS^{35}SO_3H$ and minor one (19%) corresponding to authentic $^{35}SO_3^{2-}$ or $^{35}SO_4^{2-}$, were found in the chromatogram of the control filtrate (Fig. 1A). On the other hand, in the NEM-treated filtrate the peak corresponding to authentic $^{35}SO_3^{2-}$ and/or $^{35}SO_4^{2-}$ completely disappeared and a single peak was observed (Fig. 1B). In order to identify the metabolite of $PaS^{35}SO_3H$, the radioactive fractions (8—12) in Fig. 1B were pooled and evaporated repeatedly to dryness by adding water to remove NH_4HCO_3. The residue was further desalted (phosphate) on a column of Amberlite XAD-2

9) H. Nakamura and Z. Tamura, *Bunseki Kagaku*, 22, 1356 (1973).
10) G.A. Bray, *Anal. Biochem.*, 1, 279 (1960).

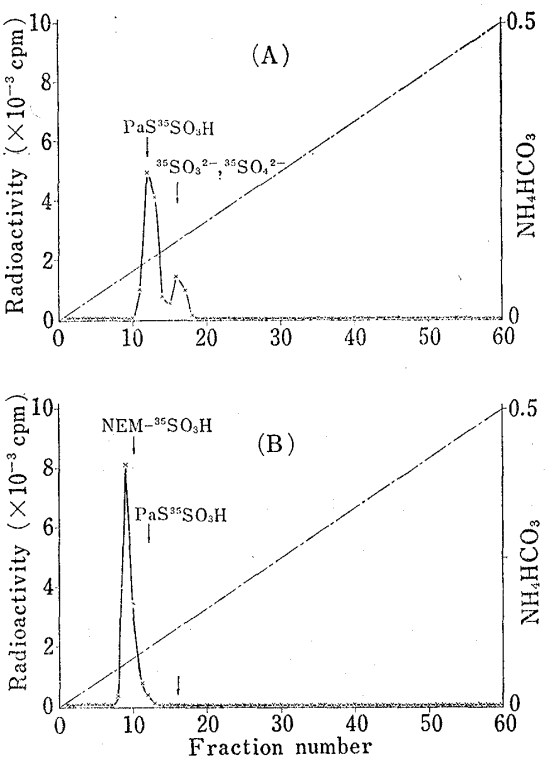


Fig. 1. Elution Patterns from QAE-Sephadex Columns of The NEM-treated Filtrate

(A): the control filtrate,
(B): the NEM-treated filtrate.
The arrows indicate the elution positions of authentic compounds.

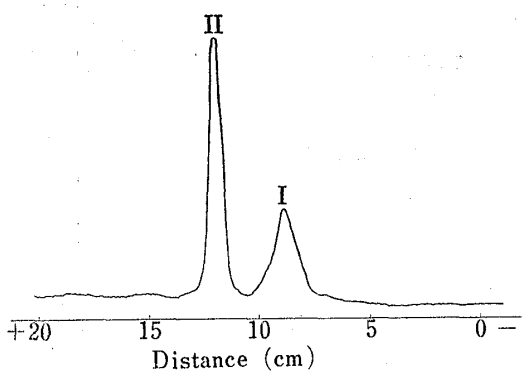


Fig. 2. Scanning Radiochromatogram of The Eluate from Amberlite XAD-2 Column

Table I. Paper Electrophoretic Behaviour of The Eluate

Sample	Distance (cm)
Eluate from Amberlite XAD-2	+ 8.7, +11.6
$(PaS^{35}SO_3)_2Ca$	+ 8.8
NEM+$Na_2{}^{35}SO_3$	+11.7, +19.7[a]
$Na_2{}^{35}SO_4$	+19.9
PaSS[b]	0

1M HCOOH (pH 2), 11.8 v/cm, 2 hr
a) trace *b*) detected by bioautography

Table II. Identificaion of NEM-$^{35}SO_3H$ by Recrystallization

Solvent system	Specific activity[a] (cpm/mg)
1 st H_2O/EtOH	364
2nd H_2O/MeOH	345
3rd H_2O/acetone/ether	341

a) The theoretical value is 376 cpm/mg.

(0.9×22 cm) by washing with water (42 ml). The adsorbed compounds were eluted with MeOH (42 ml), condensed *in vacuo* and analyzed by PEP in 1M HCOOH (pH 2).

As shown in Fig. 2, the paper electrophoretogram of the eluate gave two radioactive peaks (I & II), which were tentatively identified as $PaS^{35}SO_3H$ and NEM–$^{35}SO_3H$ respectively in the comparison of their mobilities with those of authentic compounds (Table I). Further valid evidence for identity of NEM–$^{35}SO_3H$ in the sample was given by extracting the area corresponding to peak II from the paper and recrystallizing with authentic cold NEM–$SO_3Na\cdot 1/2\ H_2O$. The result of successive three times of crystallization (Table II) demonstrates the presence of NEM–$^{35}SO_3H$ in the NEM-treated culture filtrate, and consequently that of $H^{35}SO_3^-$ in the original filtrate as an exclusive radioactive metabolite of $PaS^{35}SO_3H$ in *B. bifidum* N4.

Identification of Bisulfite by HCHO-PABA Treatment

B. bifidum N4 grown overnight in 30 ml of the preculture medium[4] was washed three times with 0.9% NaCl and suspended in 6 ml of CySS(−)MGM containing $(PaS^{35}SO_3)_2Ca$ (1.75×10^5 cpm). After incubation for 60 min at 37°, the culture was centrifuged for 5 min at 4000 rpm and the supernatant was filtered through a membrane filter (Millipore, type HA, 0.45 μ, 25 mm i.d.). Four ml of the resulting filtrate was added with 0.5 ml of formalin (37%

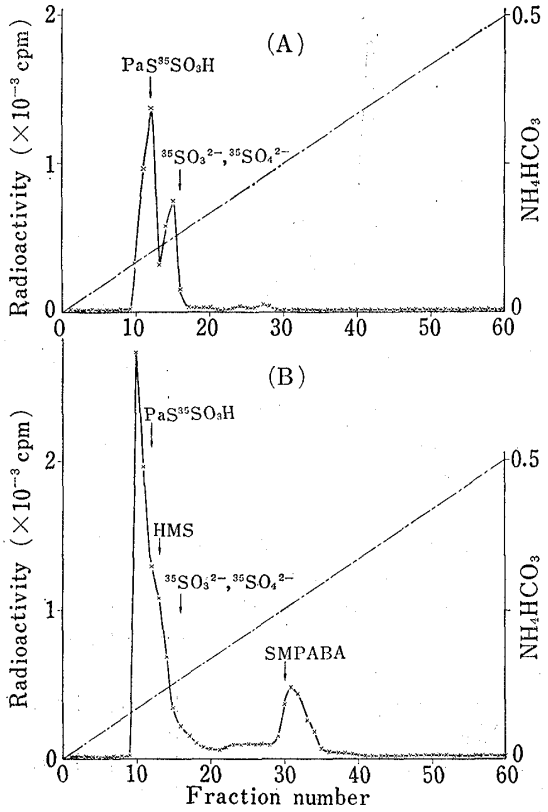


Fig. 3. Elution Patterns from QAE-Sephadex Columns of the HCHO-PABA-treated Filtrate

(A): the control filtrate
(B): the HCHO-PABA-treated filtrate
The arrows indicate the elution positions of authentic compounds.

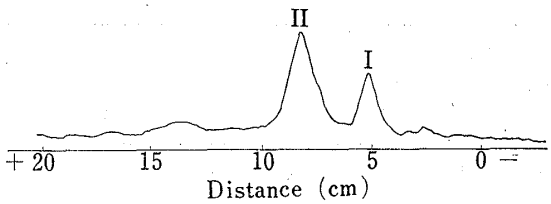


Fig. 4. Scanning Radiochromatogram of The Radioactive Fractions

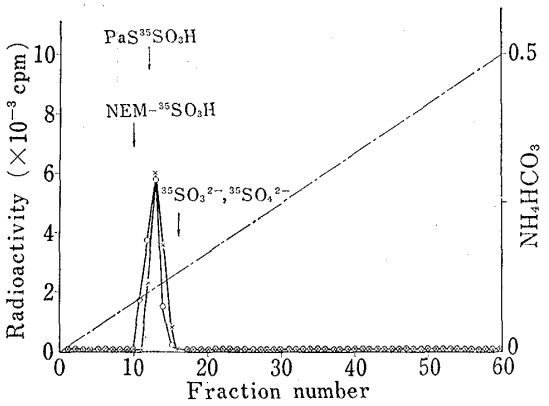


Fig. 5. Elution Patterns from QAE-Sephadex Column of the Reaction Mixtures

—×—: $(PaS^{35}SO_3)_2Ca$+CySS(−)MGM
—○—: $(PaS^{35}SO_3)_2Ca$+CySS(−)MGM+NEM
The arrows indicate the elution positions of authentic compounds.

Table III. Paper Electrophoretic Behaviour of The Radioactive Fractions

Sample	Distance (cm)
frs. 29—34	+ 5.1, +8.2
PABA[a]	− 3.1
HCHO+$Na_2{}^{35}SO_3$	+12.4, +19.9
HCHO+$Na_2{}^{35}SO_3$+PABA	+ 8.2
$Na_2{}^{35}SO_4$	+19.9
PaSS[b]	0

1M HCOOH (pH 2), 11.8 v/cm, 2 hr
a) Detected as a purple spot by the mixed fluorescent materials method.
b) detected by bioautography

Table IV. Identification of SMPABA-^{35}S by Recrystallization

Solvent system	Specific activity[a] (cpm/mg)
1st H_2O/EtOH	287
2nd H_2O /dioxane	263
3rd H_2O/acetone	275

a) The theoretical value is 291 cpm/mg.

HCHO) and then 100 mg of PABA which was suspended in 1 ml of water and adjusted to pH 7.5 with 2N NaOH, by which the pH of the filtrate increased to 7.0 from 4.6. After adjusting the final pH to 7.65 with 2N NaOH, the mixture was incubated for 18 hr at 37° and analyzed by a column of QAE-Sephadex A-25 (OH form, 0.8×12 cm). On the other hand, two ml of the filtrate through the membrane filter was, as a control, analyzed by the column chromatography without any treatment.

As shown in Fig. 3A, the column chromatography of the control filtrate gave two peaks, perhaps due to $PaS^{35}SO_3H$ and $^{35}SO_3{}^{2-}$ and/or $^{35}SO_4{}^{2-}$. However, the peak corresponding to $^{35}SO_3{}^{2-}$ and/or $^{35}SO_4{}^{2-}$ completely disappeared after treating the filtrate with HCHO and PABA, and in turn a new peak whose elution position coincided with that of authentic

SMPABA appeared (Fig. 3B). The fractions 29 to 34 in Fig. 3B were pooled, evaporated to dryness repeatedly by adding water to remove NH_4HCO_3 and analyzed by PEP in 1M HCOOH (pH 2).

As shown in Fig. 4, the radioactivity distributed in two peaks (I & II). The peak II was tentatively identified as SMPABA with the data on mobilities of authentic compounds listed in Table III, however, the peak I was not identified. The identity of SMPABA in the sample was finally confirmed by the data obtained by recrystallization of the compound in the peak II (Table IV).

Denial of the Formation of Bisulfite through Other Routes

A possibility of the chemical formation of $H^{35}SO_3^-$ from $PaS^{35}SO_3H$ was examined on the basis whether NEM–$^{35}SO_3H$ was formed or not. $(PaS^{35}SO_3)_2Ca$ (1.46×10^5 cpm) was incubated in the absence of the bacteria for 60 min at 37° in 4 ml of CySS(−)MGM and chromatographed on the column of QAE-Sephadex A-25 (OH form, 0.8×12 cm) with or without the NEM treatment. As shown in Fig. 5, although the peak of $^{35}SO_3^{2-}$ was not observed in the chromatogram of the sample without the NEM treatment, to make sure of absence of $H^{35}SO_3^-$, the radioactive fractions of the NEM-treated sample were pooled, desalted on a column of Amberlite XAD-2 (0.9×22 cm) and analyzed by PEP (1M HCOOH, pH 2) as described above. Only $PaS^{35}SO_3H$ was detected and no trace of NEM–$^{35}SO_3H$ was detected. These results deny the chemical degradation of $PaS^{35}SO_3H$ to $H^{35}SO_3^-$ by CySS(−)MGM or NEM.

Furthermore, any degree of transformation of $^{35}SO_4^{2-}$ to $H^{35}SO_3^-$ by *B. bifidum* N4 did not occur under the culture conditions employed, which was confirmed by the absence of NEM–$^{35}SO_3H$ in the culture filtrate of the strain incubated for 60 min at 37° with $Na_2{}^{35}SO_4$ (2×10^5 cpm) in 6 ml of CySS(−)MGM omitted with cold SO_4^{2-}.

Discussion

In a previous communication,[3] we assumed the formation of $^{35}SO_4^{2-}$ from $PaS^{35}SO_3H$ by *B. bifidum* N4. However, the conclusion must be revised in the present paper. The reason why we made a mistake is perhaps that SO_3^{2-} was not distinguished from SO_4^{2-} by QAE-Sephadex column chromatography. In addition to this, oxidation of SO_3^{2-} to SO_4^{2-} during the overnight fractionation and the successive analytical procedures might be considered.

$CyS^{35}SO_3H$ was substantially not formed[3] through cultivation of *B. bifidum* N4 with $(PaS^{35}SO_3)_2Ca$ in the basal medium[4] containing L-cystine, however, a quantitative formation of $CyS^{35}SO_3H$ was observed when the acidic culture filtrate (usually pH 4.4 to 4.6 due to acetic and lactic acids) was neutralized and incubated at 37° for the formation reactions of NEM–SO_3Na and SMPABA (Fig. 6). Therefore, L-cystine was omitted from the assay medium in this work. Although the amino acid is essential for the growth of *B. bifidum*, the production of $H^{35}SO_3^-$ from $PaS^{35}SO_3H$ was not affected by omitting it.

The production of $H^{35}SO_3^-$ as a sole radioactive metabolite of $PaS^{35}SO_3H$ indicates that

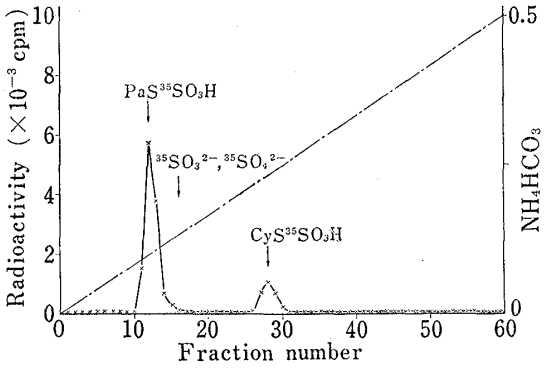


Fig. 6. Elution Pattern from a QAE-Sephadex Column of the Culture Filtrate obtained by incubating *B. bifidum* N4 with $(PaS^{35}SO_3)_2Ca$ in the Basal Medium Containing L-Cystine

The culture and the successive procedures were carried out in the same manner as the NEM treatment described in the text, except for use of the basal medium instead of CySS(−)MGM. The arrows indicate the elution positions of authentic compouwnds. $CyS^{35}SO_3H$ was synthesized by sulfitolysis of L-cystine with $Na_2{}^{35}SO_3$.

the cleavage of –SS– bond in the molecule proceeds by a reductive process and not by a hydrolytic one.

Acknowledgement The authors are indebted to all the staffs of central analytical laboratory of this Faculty for elemental analyses and spectral measurements. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, to which their gratitude are also expressed.