JOURNAL OF BACTERIOLOGY, Apr. 1969, p. 62-68
Vol. 98, No. 1

Biochemical Studies of Bacterial Sporulation and Germination

XII. A Sulfonic Acid as a Major Sulfur Compound of *Bacillus subtilis* Spores

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Received for publication 27 December 1968

A sulfonic acid found to be a major constituent of spores of *Bacillus subtilis* was provisionally identified as 3-L-sulfolactic acid. This compound was completely absent from vegetative cells during growth, but large amounts accumulated in sporulating cells just before the development of refractile spores. Essentially all of the accumulated sulfolactic acid was eventually incorporated into the mature spore, where it may represent more than 5% of the dry weight of the spore. Germination resulted in the rapid and complete release into the medium of unaltered sulfolactic acid. This compound was not found in spores of *Bacillus megaterium*, *B. cereus*, or *B. thuringiensis*.

Many of the macromolecules of the spore resemble, in their nature and relative abundance, those of the vegetative cell (8). Is this also true of small molecules? Dipicolinic acid provides an immediate exception. This compound is unique to the spore and comprises about 10% of its dry weight (6). However, additional examples of this type have not been reported nor is it known whether any small cellular compounds are excluded from the spore. The importance of small molecules in the conduct and regulation of metabolism and the contributions that such molecules may make to the structural and physiological features of cells and spores warrant their intensive investigation. An opportunity to explore in this direction was provided by our observations that the ^{35}S-sulfate taken up by sporulating *Bacillus subtilis* and deposited in spores was almost entirely in a small molecule which was not found in vegetative cells (J. A. Spudich, Ph. D. Thesis, Stanford Univ., Stanford, Calif., 1967). This report describes the properties of this compound and its provisional identification as 3-sulfolactic acid. Studies of the nature and distribution of phosphate compounds and amino acids are discussed elsewhere. (D. L. Nelson et al., Spores IV, *in press*).

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MATERIALS AND METHODS

B. subtilis strain SB 133, a phenylalanine auxotroph, was obtained from the culture collection of the Department of Genetics at Stanford University School of Medicine. *B. megaterium* strain QM B1551 was a gift from H. S. Levinson, U.S. Army Natick Laboratories, Natick, Mass. *B. cereus* T originated from the University of Wisconsin and was kindly provided by J. C. Vary. *B. thuringiensis* T34 was obtained from P. C. Fitz-James, University of Western Ontario, Canada.

^{35}S-sulfate ($^{35}SO_4^{2-}$) was obtained from Nuclear Science and Engineering Corp. and from New England Nuclear Corp. ^{35}S-sulfur dioxide was purchased from New England Nuclear Corp. L-Cysteic acid and taurine were obtained from Calbiochem. D-Cystine and phosphoenolpyruvate were obtained from the Sigma Chemical Co.

Reference compounds. ^{35}S-cysteic acid was obtained from the protein fraction of either *Chlorella vulgaris* or a species of *Aegialitis* (mangrove) grown in the presence of $^{35}SO_4^{2-}$. The protein fraction, after oxidation with performic acid, was hydrolyzed with 6 N HCl for 18 hr at 110 C. Cysteic acid was then purified on thin-layer chromatograms, with propanol-concentrated ammonia-water (6:3:1, v/v) as the solvent system.

^{35}S-sulfoacetic acid was prepared from phosphoenolpyruvate and ^{35}S-sulfur dioxide as described by Lehmann and Benson (9).

^{35}S-sulfopropanediol was synthesized by reacting

1-iodo-1-deoxyglycerol [prepared according to Baer and Fischer (1)] with ^{35}S-bisulfite as described for the synthesis of related compounds by Lehmann and Benson (9).

^{35}S-sulfoacetaldehyde was prepared from ^{35}S-cysteic acid by reaction with ninhydrin at *p*H 1, as described by Shibuya (Ph.D. Thesis, Pennsylvania State University, University Park, 1960).

^{35}S-isethionic acid was synthesized from ^{35}S-sulfoacetaldehyde by reduction with sodium borohydride.

^{35}S-sulfolactic acid was prepared from ^{35}S-cysteic acid by deamination with nitrous acid, as described by Shibuya (Ph.D. Thesis, Pennsylvania State University, University Park, 1960).

^{35}S-sulfolactaldehyde was obtained as a contamination in the preparation of ^{35}S-sulfolactic acid as described above.

L-Sulfolactic acid was synthesized from cysteic acid as follows. L-Cysteic acid (1 g) was dissolved in 10 ml of water, acidified with HCl to *p*H 1, and cooled in ice. A solution of 0.5 g of sodium nitrite in 5 ml of water was added dropwise over a period of 5 min. After 30 min at 0 C, the solution was kept at room temperature for 2 hr. Dowex-50 (H^+) was then added to remove all sodium ions. The solution was adjusted to *p*H 7 with $Ba(OH)_2$, and the barium salt of sulfolactic acid was precipitated by cooling overnight in a refrigerator. This barium salt was again converted into the acid form by suspending in water and mixing with Dowex-50(H^+). The sulfolactic acid was then neutralized with NaOH solution and crystallized from water-ethyl alcohol. This gave L-sulfolactic acid (sodium salt) as a white crystalline material with a melting point >250 C.

D-Sulfolactic acid was prepared in the same way as described for the L-isomer, starting from D-cysteic acid derived from D-cystine by performic acid oxidation.

Growth and sporulation. The bacteria were grown in a supplemented nutrient broth, containing 0.1% glucose, at 37 C with vigorous aeration on a rotary shaker as described previously (16) or in a defined medium at 30 C (4). When $^{35}SO_4^{2-}$ was added, the sulfate salts normally supplementing the medium were usually replaced by the corresponding chlorides.

Sporulation occurred to the extent of about 90%, as judged in the phase-contrast microscope by the appearance of refractile forms inside the sporulating cells. The maximal number of refractile forms was observed about 9 hr after the end of exponential growth. By 24 hr, most vegetative forms had lysed and the spores had been released into the medium. Growth was followed turbidimetrically with a Klett-Summerson colorimeter as described previously (16). Spores were counted with phase-contrast optics in a Petroff-Hauser chamber.

Purification of the spores. Spores were freed from vegetative forms by incubating 100 mg of wet cells in 5 ml of water, containing 1 mg of lysozyme, at 37 C for 20 min. The spores were then purified by successive washings at 25 C with 10 ml each of 0.5% sodium dodecylsulfate, 1 M NaCl, and 0.14 M NaCl and by several washings with water. More than 95% of the spores were still refractile after this treatment.

Extraction of lysozyme-sensitive cells. The cell pellets from 1-ml samples of a culture were suspended in 1 ml of water containing 0.5 mg of lysozyme and were incubated for 20 min at 37 C. Trichloroacetic acid (0.1 ml of a 50% solution) was added to precipitate protein. $(NH_4)_2SO_4$ (3 μmoles) and $BaCl_2$ (5 μmoles) were then added to the trichloroacetic acid supernatant fluids to precipitate $^{35}SO_4^{2-}$. The final supernatant fluids were used as cell extracts and were reduced in volume by evaporation when necessary.

Extraction of spores. Spores were usually extracted by sonic oscillation with a Branson Sonifier as described previously (16); however, good release of the ^{35}S-compound was also achieved either by disruption with a colloid mill (13) or by heating intact spores at 100 C for 10 min.

Preparation of ^{35}S-labeled spore extracts. *B. subtilis* was grown and sporulated in 100 ml of the supplemented nutrient broth containing about 3 mc of $^{35}SO_4^{2-}$. After 23 hr (Fig. 2), spores were harvested and purified. These cleaned spores, containing 3% of the total ^{35}S of the culture medium, were suspended in 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, *p*H 7.8, and were extracted by sonic oscillation. In the synthetic medium, altered to contain 40 mM SO_4^{2-}, the uptake of $^{35}SO_4^{2-}$ from the medium into purified spores was about 20%.

Methods for characterization of the ^{35}S-compound. For paper chromatography, the descending technique, Whatman no. 4 paper, and the following solvent systems were used: (i) phenol-water (100:40, w/w); (ii) *n*-butyl alcohol-propionic acid-water (142:71:100, v/v).

In two-dimensional chromatography, solvent (i) was used in the first direction and (ii) in the second; the chromatograms were dried at room temperature.

For thin-layer chromatography (TLC), silica gel plates (Silica Gel G, purchased from Merck) were used with *n*-propanol-concentrated ammonia-water (6:3:1, v/v) as the solvent system.

For low-voltage electrophoresis, we used an apparatus from Research Specialities Co. (model E800-2B) and Whatman no. 1 paper (15 v/cm for 2 to 2.5 hr). Indigotetrasulfonate (a blue dye) was used as a marker, and all mobilities are expressed relative to it. The buffer systems used for electrophoresis were: (i) pyridine-acetic acid-water (1:10:189, v/v), *p*H 3.6; (ii) citric acid-phosphate (3.7 mM citric acid, 13 mM sodium phosphate), *p*H 6.0.

For detection of compounds on chromatograms and electropherograms, we used the following methods: (i) reaction with ninhydrin (0.5% in water-saturated *n*-butyl alcohol) for amino-containing compounds; (ii) charring with 30% H_2SO_4 on TLC for all compounds; and (iii) autoradiography with single-coated X-ray film (Kodak; blue sensitive).

All radioactivity measurements were made with a Nuclear-Chicago scintillation counter and the solvent of Bray (2).

RESULTS

Properties of the ^{35}S-compound isolated from spores of B. subtilis. Sonic treatment or boiling water extracted 64% of the ^{35}S from spores pre-

pared in nutrient broth and 60% of the ^{35}S from spores grown in the synthetic medium. Almost all of the ^{35}S in these extracts (93 to 98%) was soluble in 1 M trichloroacetic acid and appeared to represent a single species (*see below*).

Less than 4% of the ^{35}S was precipitated with excess $BaCl_2$ in 0.25 N HCl before or after heating at 100 C for 1 hr either in 1 N HCl or in 1 N NaOH. Therefore, sulfate is not generated from the ^{35}S-compound by these treatments. At *p*H 6, more than 94% of the ^{35}S was precipitated with $BaCl_2$ (Table 1).

G-25 Sephadex filtration indicated that the ^{35}S-compound has a molecular weight considerably lower than 5,000, but greater than that of KCl. Only a single peak of ^{35}S, equal in sharpness to that of the KCl, was observed.

The ^{35}S-compound was not retained on Dowex-50 at *p*H 2, indicating that it is neutral or negatively charged at this *p*H. Therefore, its elution from Dowex-1 was compared to that of sulfate. The ^{35}S-compound was not eluted by successive washings with 2 bed-volumes each of 0.01, 0.05, or 0.10 M HCl; it was eluted by 2 bed-volumes of 0.10 M HCl plus 0.2 M NaCl as a single, symmetrical peak preceding the elution of sulfate by 1 bed-volume. The electrophoretic mobility of the ^{35}S-compound was found to be greater than those of any of the nucleoside monophosphates, adenosine diphosphate, and adenosine triphosphate (ATP), but less than that of sulfate. The ^{35}S-compound migrated as a single species in all experiments, and more than 86% of the ^{35}S was recovered from the electropherograms.

In summary, the ^{35}S-compound is a major sulfur constituent of spores which can easily be extracted as a homogeneous species, as judged by its elution from Dowex-1 and Sephadex and its migration by electrophoresis. The failure to release sulfate from the compound by treatment with HCl or NaOH and its behavior as a strong acid suggested the presence of a sulfonic acid group.

Identification of the ^{35}S-compound as 3-sulfolactic acid. To identify this compound, some ^{35}S-sulfonic acids and related derivatives were synthesized and were subjected to the following treatments: paper chromatography (two-dimensional); electrophoresis; TLC; reaction with 2,4-dinitrophenylhydrazine; treatment with acid and treatment with alkali; acetylation with acetic anhydride; methylation with methanolic hydrochloric acid.

The ^{35}S-compound had an R_F of 0.09 in the first direction and 0.17 in the second direction on two-dimensional paper chromatography (Table 2). These figures were closest to those found for cysteic acid (0.09 and 0.13) and sulfolactic acid (0.09 and 0.17). At *p*H 6, the electrophoretic mobility of the ^{35}S-compound relative to indigotetrasulfonate was 1.15 (Table 2). This value was closest to those for sulfoacetic acid (1.18) and sulfolactic acid (1.15). At *p*H 6.0, sulfoacetic acid showed a relative mobility different from that of the unknown spore component (1.84 versus 1.64).

TLC gave a clear separation of the ^{35}S-compound from cysteic acid and sulfopropanediol, but not from sulfolactic acid (Fig. 1). The ^{35}S-compound did not react with 2,4-dinitrophenylhydrazine, indicating the absence of a keto-function, and is therefore not a keto acid derivative such as sulfopyruvic acid.

TABLE 1. *Properties of the ^{35}S-compound*[a]

Determination	Finding
Amount	>70 μmoles/liter of culture
Solubility in acid	93–98%
Precipitability by Ba^{++} at *p*H 1	
Untreated	<4%
Treated with HCl or NaOH at 100 C	<4%
Precipitability by Ba^{++} at *p*H 6	>94%
Filtration on G-25 Sephadex	Molecular weight, <5,000
Dowex 1-Cl chromatography	Eluted after ATP, before SO_4^{2-}
Electrophoretic mobility	>ATP; <SO_4^{2-}

[a] Spores grown in supplemented nutrient broth containing $^{35}SO_4^{2-}$ were extracted as described above. For filtration on G-25 Sephadex, the column (1.3 cm^2 by 32 cm; 20- to 80-μm beads) was equilibrated with buffer (20 mM potassium phosphate, *p*H 7.4), and the sample (0.5 ml of extract plus 0.4 ml of a mixture of 2% KCl and dextran blue 2000) was filtered through the column at a flow rate of 5 ml per hr; $AgNO_3$ was used to test for Cl^-. For Dowex 1-Cl chromatography, a sample (3 ml) of extract was made 10% in trichloroacetic acid and centrifuged; the supernatant fluid was adjusted to *p*H 7 with NaOH, mixed with Na_2SO_4 (30 μmoles), and loaded onto a Dowex 1-Cl^--X2 column (0.78 cm^2 by 15 cm, 50 to 100 mesh, 0.8 meq per ml). After elution with HCl, SO_4^{2-} was estimated turbidimetrically at 660 nm after precipitation with $BaCl_2$ at acid *p*H. For determination of electrophoretic mobility, Whatman 3 MM paper was used at 5 to 20 ma, 800 to 1,200 v, for 1 to 3 hr in 0.01 M or 0.05 M sodium citrate, *p*H 3.3.

TABLE 2. *Chromatographic and electrophoretic values of the ^{35}S-compound and related compounds*[a]

Compound	Chromatography		Electrophoresis	
	R_F in solvent (i)	R_F in solvent (ii)	Relative mobility at pH 3.6	Relative mobility at pH 6.0
^{35}S-compound	0.09	0.17	1.15	1.64
Sulfolactic acid	0.09	0.17	1.15	1.64
Cysteic acid	0.09	0.13	0.80	0.69
Sulfopropanediol	0.41	0.26	0.90	0.80
Sulfoacetic acid	0.16	0.18	1.18	1.84
Sulfoacetaldehyde	0.18	0.30	0.96	
Sulfolactaldehyde	0.17	0.18	0.83	
Isethionic acid	0.54	0.50	0.56	
Inorganic sulfate	0.08	0.10	1.70	1.95
Indigo tetrasulfonate			1.00	1.00
^{35}S-compound, acetylated		0.27	1.15	
^{35}S-compound, methylated	0.09	0.36	0.80	
Acetyl sulfolactic acid		0.27	1.15	
Methyl sulfolactic acid	0.09	0.36	0.80	

[a] For acetylation, samples were evaporated in vacuo, dried over P_2O_5, dissolved in 0.02 ml of dry pyridine, mixed with 0.01 ml of acetic anhydride, and heated at 40 C for 4 hr. For methylation, dried samples were dissolved in 0.5 ml of 10% anhydrous HCl in methanol and heated at 50 C for 1.5 hr.

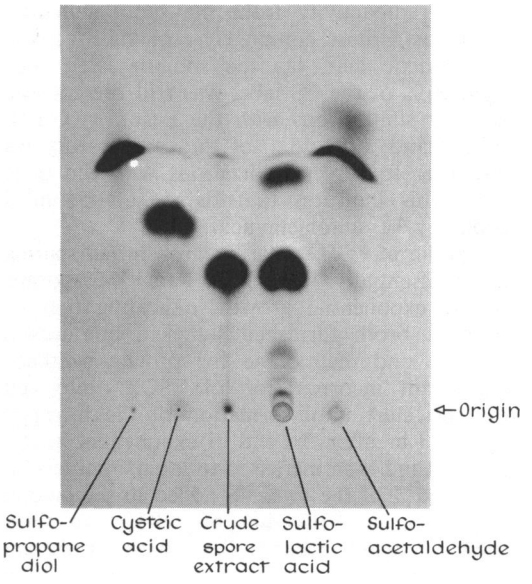

FIG. 1. *TLC autoradiogram of the ^{35}S-compound and related sulfonic acids. Note that the synthetic sulfolactic acid contains an impurity (sulfolactaldehyde). The sulfopropanediol spot is one-half the distance to the solvent front.*

Co-chromatography and co-electrophoresis of the ^{35}S-compound with sulfolactic acid gave in all cases one single spot, indicating that the ^{35}S-compound is very likely identical with sulfolactic acid.

In attempts to identify further the ^{35}S-compound with sulfolactic acid, the compounds were subjected to the treatments outlined above, and the resulting derivatives were compared in each case according to the procedures described in Materials and Methods.

(i) Treatment with acid (1 N HCl for 1 hr at 100 C) or alkali (1 N KOH for 1 hr at 100 C) did not alter the chromatographic or electrophoretic properties of the ^{35}S-compound or sulfolactic acid.

(ii) Acetylation with acetic anhydride and pyridine converted both compounds to acetyl derivatives, which had identical electrophoretic mobilities relative to indigotetrasulfonate and identical R_F values on chromatograms (Table 2).

(iii) The methyl esters (at the carboxyl group), formed by reaction with methanolic hydrochloride, had identical relative electrophoretic mobilities and identical R_F values on paper chromatograms (Table 2).

From these experiments, it appears that the spore component is identical with sulfolactic acid. It must be emphasized, however, that the possibility that the ^{35}S-compound is a homologue of sulfolactic acid with one or more additional CH_2-groups (namely, sulfohydroxybutyric acid) has not been rigorously excluded, and its identity with sulfolactic acid is therefore provisional.

Stereochemical configuration. We recrystallized L- and D-sulfolactic acid from water-ethyl alcohol at 0 C in the presence of ^{35}S-labeled sulfolactic acid from spores.

The ^{35}S-sulfolactic acid from spores was first purified on a preparative scale on paper chromatograms, with solvent (ii), eluted with water, converted into the acid form with Dowex-50 (H^+), and then neutralized with NaOH. The sodium salts of L- and D-sulfolactic acid were synthesized as described above. The formation of α-hydroxy acids from the corresponding amino derivatives, with nitrous acid as deaminating agent, proceeds via an S_N-1 mechanism, wherein neither inversion of configuration nor appreciable racemization occurs (3).

The radioactivity from the spore sulfolactic acid was almost completely cocrystallized with L-sulfolactic acid (as the sodium salt); more than 90% of the ^{35}S label was still present after six recrystallizations with the L-isomer. On the other hand, only 15% of the radioactivity was left after four recrystallizations with the D-isomer. This indicates that the ^{35}S-compound is probably 3-L-sulfolactic acid.

Uptake of $^{35}SO_4^{2-}$ during growth and sporulation. Essentially no $^{35}SO_4^{2-}$ was incorporated during exponential growth, indicating that the nutrient broth furnished sufficient amounts of cysteine and methionine for protein synthesis. Significant incorporation of $^{35}SO_4^{2-}$ into cells did not start until formation of the forespore [about 3 hr after the end of exponential growth (5, 14)] and then increased to a maximal level of about 15% of the total ^{35}S added to the culture.

Kinetics of conversion of $^{35}SO_4^{2-}$ to forms soluble in acid and Ba^{++}. Ten samples of the culture were each separated into cells and medium by centrifugation. The amount of acid-soluble and Ba^{++}-soluble ^{35}S in the medium increased linearly throughout sporulation and reached a maximal level before a significant amount appeared in the cells (Fig. 2). The cells appeared to be totally sensitive to lysozyme until 9 hr, at which time lysozyme-resistant, refractile spores began to appear. The further appearance of refractile forms was accompanied by a concomitant decrease in lysozyme-extractable ^{35}S, the remainder being found in the spore extracts prepared by sonic treatment. Thus, the majority of the ^{35}S in the 12-hr sample was not in the mother cells, but in the spores (Fig. 2).

Analysis of the acid-soluble and Ba^{++}-soluble ^{35}S-labeled compounds in the various fractions. The ^{35}S-compounds in the medium obtained from the 6.3- to 11.3-hr samples were analyzed by TLC. There were 10 to 20 radioactive compounds, most of which were acid-labile, suggesting the presence of sulfate esters or related compounds. Less than 5% of the radioactivity in these samples was in the form of sulfolactic acid. The medium obtained from the 12- and 27-hr samples appeared to contain a somewhat higher percentage (about 20%) of sulfolactic acid, but this still represented less than 20% of the sulfolactic acid found in spores at these times (*see below*). No further attempts were made to identify the other radioactive components in the medium.

The acid-soluble and Ba^{++}-soluble ^{35}S-compounds in the lysozyme extracts obtained from the 7.5- to 11.3-hr samples included a small amount of acid-labile material (<5%; presumably sulfate esters), two unidentified products in low concentration (<5%), and two

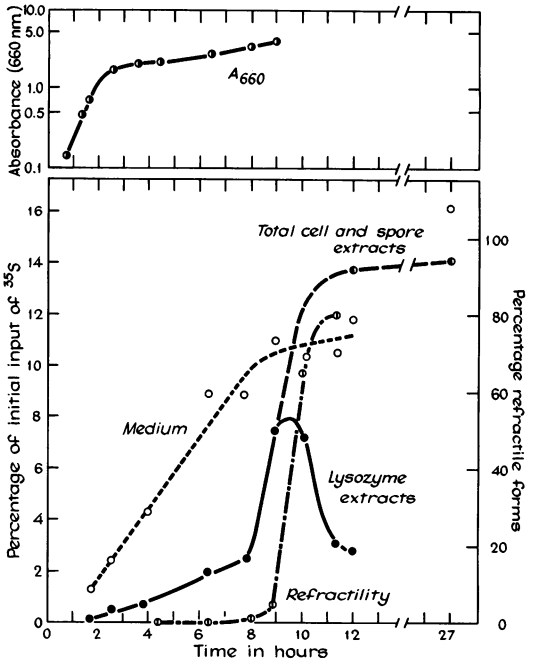

FIG. 2. *Kinetics of conversion of sulfate to sulfolactic acid and other acid-soluble and Ba^{++}-soluble forms. Cells were grown and sporulated in 100 ml of broth with $^{35}SO_4^{2-}$ (2×10^7 counts per min per ml of culture). The 2-hr point is fixed at the time when exponential growth is ended. Samples of the culture were centrifuged for 5 min at 20,000 × g to separate the cells from the medium. The sporulating cells from samples taken at 1.5 to 11.3 hr were disrupted with lysozyme. The 12-hr sample, in which most cells had a refractile spore, was treated with lysozyme to yield a mother cell extract and free spores. The spores from the 12-hr sample and the free spores from the 27-hr sample were purified and extracted. The samples of supernatant medium and of lysozyme-cell extracts were all treated with trichloroacetic acid and $BaCl_2$ before measuring the ^{35}S.*

principal compounds (TLC). Sulfolactic acid accounted for about 75% of the ^{35}S. The second main compound (about 15%) was identified as cysteic acid (paper chromatography, TLC, and electrophoresis at two different *p*H values). A negative reaction with 2,4-dinitrophenylhydrazine indicated the absence of sulfopyruvic acid.

Treatment with lysozyme at 12 hr released the contents of that part of the sporulating cell which surrounds the developing spore. The general pattern of ^{35}S-compounds in extracts prepared in this way was similar to that found in the lysozyme extracts discussed above. The amount of sulfolactic acid, however, did not account for more than 50% of the total ^{35}S and represented only about 10% of that found in the

spores at this time (*see below*). The lower percentage of ^{35}S-sulfolactic acid in this lysozyme extract (12 hr), as compared to earlier lysozyme extracts, probably resulted from an increased resistance of the developing spore to lysozyme. For that matter, the ^{35}S-sulfolactic acid found in this lysozyme-cell extract (12 hr) may have come from forespores which had not yet developed resistance to lysozyme.

Both spore preparations (12- and 27-hr samples) contained, after purification and disruption by sonic oscillation, sulfolactic acid exclusively as the soluble ^{35}S-compound. In this experiment, about 75% of the radioactivity of the spores was extracted.

After sonic disruption, performic acid treatment, and acid hydrolysis, more than 80% of the ^{35}S in the insoluble fraction (spore coats) was recovered as cysteic acid, as identified by TLC and electrophoresis.

From the foregoing analyses and the data shown in Fig. 2, the synthesis of sulfolactic acid is seen to occur late in sporulation, just before (within 1 hr of) the appearance of refractile forms.

Amount of sulfolactic acid in spores. The amount of sulfolactic acid in spores could be estimated from the specific radioactivity of $^{35}SO_4^{2-}$ in the sporulation medium, and from the amount of radioactivity in sulfolactic acid from spores grown in that medium.

The specific radioactivity of $^{35}SO_4^{2-}$ in the supplemented nutrient broth was calculated, from the known amount of carrier sulfate added, to be $<10^7$ counts per min per μmole. About 95% of the ^{35}S extracted from the spores (7×10^7 counts/min from 100-ml culture) was in the sulfolactic acid. Thus, at least 7 μmoles of the sulfolactic acid was extracted from spores from 100 ml of culture (70 μmoles per liter). The protein extractable from spores (about 5% of the ^{35}S extracted) amounts to about 28 mg per liter of culture (16) and is about 2% S by weight. Thus, there is about 17.5 μmoles of extractable protein sulfur per liter of culture having a specific radioactivity of about 2×10^6 counts per min per μmole. Since the dry weight of spores from 1 liter of nutrient broth is about 400 mg, the percentage (dry weight) of sulfolactic acid is about: $[(0.171 \text{ mg } \mu\text{mole}^{-1})\ (70 \ \mu\text{mole liter}^{-1})/400 \text{ mg liter}^{-1}] \times 100 = 3.0\%$. From the known specific radioactivity of SO_4^{2-} in the synthetic medium and a direct count of the spores, we could calculate that spores in this medium contained 1.6 μmoles of sulfolactic acid per 10^{10} spores, comprising about 5.5% of their dry weight. This is equivalent to 55 μmoles of sulfolactic acid per liter of culture.

Fate of sulfolactic acid during germination. After 2 hr of germination, 90% of the spores had darkened and about 60% of the ^{35}S was released into the medium (Fig. 3). Chromatographic and electrophoretic analyses of the ^{35}S released into the medium showed that sulfolactic acid as such was released; no other ^{35}S-labeled compounds were detected by these methods. The ^{35}S-sulfolactic acid released during germination (60% of the ^{35}S in the spore before heat-shock) accounted for about 80% of the sulfolactic acid which could be extracted from these spores by sonic disruption (75% of the ^{35}S in the spore).

Absence of sulfolactic acid from spores of other species. ^{35}S-labeled spores of *B. megaterium*, *B. cereus*, and *B. thuringiensis* were prepared by growth at 30 C in supplemented nutrient broth containing $^{35}SO_4^{2-}$. They contained a much smaller fraction of hot water-extractable ^{35}S than that in *B. subtilis* spores; two successive extractions with boiling water, each for 30 min, released less than 10% of the total ^{35}S in each case, whereas dipicolinic acid was released completely. Fractionation of these extracts by paper electrophoresis at *p*H 3.3 showed no sulfolactic acid present. Some material with the mobility of SO_4^{2-} was detected, as well as some which did not move from the origin. We conclude that spores of these species, prepared under these conditions, do not contain sulfolactic acid.

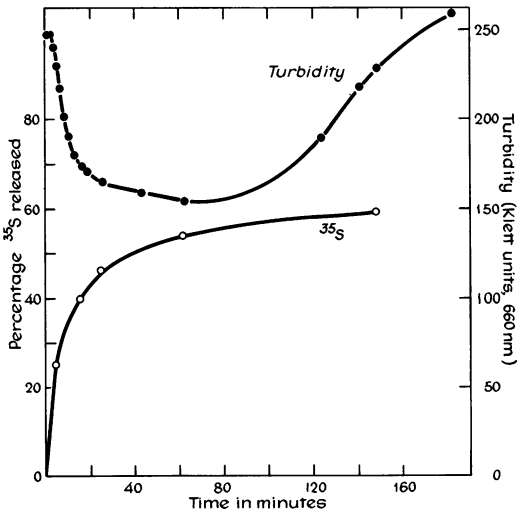

FIG. 3. *Kinetics of release of sulfolactic acid (^{35}S) during germination of spores. An aqueous suspension of spores (27-hr sample, see Fig. 2) was heat-shocked for 20 min at 70 C (a treatment that released 10% of the ^{35}S), centrifuged, and resuspended in supplemented nutrient broth at 37 C. Germination was followed turbidimetrically with a Klett-Summerson colorimeter. Identical results were obtained when samples of the suspension were mixed with trichloracetic acid (5% final concentration) before sedimentation of the germinating spores by centrifugation.*

DISCUSSION

A sulfonic acid, very probably 3-L-sulfolactic acid, is present in large amounts in spores of *B. subtilis* and is virtually the only small sulfur compound present. Sulfolactic acid was not detected in vegetative cells before sporulation. This compound has been described previously as a trace component in some algal species (15). In general, very little is known about the occurrence, formation, and metabolism of sulfonic acids in microorganisms. Cysteic acid is found in some microorganisms (12) and taurine is a cell wall constituent in a *B. subtilis* variant (7). Some soil bacteria (*Flavobacterium* species) can utilize organic sulfonic acids as carbon sources. Thus, methyl sulfoquinovoside is converted to cysteic acid, sulfoacetic acid, and sulfate, whereas the only hydrolysis product of sulfolactic acid identified is sulfate (11); sulfopropanediol is converted into several other sulfonic acids (10).

The biosynthetic pathway for sulfolactic acid is entirely unknown. One possible route is by reduction of sulfopyruvic acid, which may in turn be derived from cysteic acid by oxidation or transamination. Although sulfopyruvic acid was not detected at any time during growth or sporulation, its role as an intermediate may have been obscured by its ready conversion to sulfolactic acid. Conversion of ^{35}S-cysteic acid to taurine and sulfolactic acid (recrystallized as a guanidine salt to constant specific radioactivity with authentic sulfolactic acid) by cell-free preparations of *Neurospora crassa* has been observed (R. L. Metzenberg, *personal communication*). A firm conclusion as to whether cysteine serves as the precursor of the sulfolactic acid in the *B. subtilis* spore requires additional data. The specific radioactivity of the cysteine sulfur in the extractable protein was 2×10^6 counts per min per μmole, whereas that of the sulfolactic acid might have been as high as 10^7. Assuming the latter value, sulfolactic acid could not be derived from the same cysteine pool that serves in the biosynthesis of the protein. Had the sulfolactic acid possessed the same specific radioactivity as the cysteine in protein, then sulfolactic acid would represent 15% rather than 3% of the spore dry weight.

The function of sulfolactic acid is as obscure as its origin. Our experiments suggest that either it is synthesized in the forespore just before the appearance of refractility or, if synthesized in the mother cell, it is specifically absorbed by the developing spore. It seems plausible that sulfolactic acid is designed to play a part in shaping some of the physiological and structural attributes of the spore. It is noteworthy that, like dipicolinic acid, its structure suggests a strong chelating capacity. The virtually complete release of the spore's sulfolactic acid early in germination makes it unlikely that it plays any role during outgrowth.

ACKNOWLEDGMENTS

The encouraging interest and guidance of Andrew A. Benson are highly appreciated.

This investigation was supported by Public Health Service research grant GM 12310 to the University of California, San Diego, and by National Science Foundation grant GB-4082 to A.K. D.L.N. is a National Science Foundation predoctoral fellow.

ADDENDUM IN PROOF

In a paper recently called to our attention [H. Kadota and A. Uchida, J. Agr. Chem. Soc. Japan 41:485–491 (1967)], studies of acid-soluble sulfur compounds in another strain of *B. subtilis* (Marburg, ATCC 6051) were reported. The only sulfur-containing small molecules found in dormant spores of this strain were cysteine and methionine, and they were present at levels far below that of sulfolactic acid in *B. subtilis* SB133.

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