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Sporulation in *Bacillus subtilis*

THE APPEARANCE OF SULPHOLACTIC ACID AS A MARKER EVENT FOR SPORULATION

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1. The synthesis of sulpholactic acid in sporulating cultures of *Bacillus subtilis* was studied. 2. Sulpholactic acid was first detected about 4h after the initiation of sporulation and 1h before refractility. The rate of synthesis paralleled that of the other events of sporulation examined. 3. Sulpholactic acid accounted for 1.7% of the material of the spore. 4. Because the addition of chloramphenicol in the earlier stages of sporulation inhibited formation of the compound, it is likely that the enzymes concerned are synthesized *de novo* during sporulation. 5. In asporogenous mutants only those blocked at a late stage and showing partial refractility were able to produce sulpholactic acid. This correlation makes sulpholactic acid a useful marker event in sporulation.

A number of biochemical events have been shown to occur in a temporal sequence in *Bacillus subtilis* during sporulation (Szulmajster, 1964; Schaeffer, Ionesco, Ryter & Balassa, 1965). Events such as the formation of antibiotic (Schaeffer, 1969) or dipicolinic acid (Powell, 1953) which are associated with sporulation are useful as 'markers', i.e. they help to define the stage of sporulation reached. This biochemical sequence has been correlated with the morphological events of sporulation (Kay & Warren, 1968). The same sequence of events has been shown to occur in asporogenous mutants of the same strain. In these, morphological and biochemical development are linked so that failure of development in one sequence of events arrests the other (Waites *et al.* 1970).

An aliphatic sulphonic acid identified as 3-L-sulpholactic acid has been demonstrated to occur in sporulating cultures of *B. subtilis* (Bonsen, Spudich, Nelson & Kornberg, 1969). This compound apparently constituted up to 5% of the dry weight of the spore and was not present in exponentially growing cells. The present study was undertaken to determine if the synthesis of sulpholactic acid could be considered as a spore-specific event comparable with the other sporulation events in wild-type cells. If this were so its appearance should be correlated with the morphological and biochemical stage reached by asporogenous mutants and it should depend on the proper occurrence of earlier events such as protease and alkaline phosphatase for its expression (see Waites *et al.* 1970).

MATERIALS AND METHODS

Organisms. *B. subtilis* 168 (Marburg) which requires indole or tryptophan for growth was used for most experiments. The biochemical and morphological characteristics of the asporogenous mutants derived from it have been described by Waites *et al.* (1970).

Culture. All strains used were grown in hydrolysed casein medium, and sporulation was initiated by resuspending the cells in glutamate–minimal medium as described by Sterlini & Mandelstam (1969). These will be referred to as resuspension experiments. The sulphates of the latter medium were normally replaced by the corresponding chlorides. Usually 80% refractile spores were obtained after 8h of aeration at 37°C. Unless otherwise stated all experiments were done in this resuspension medium. For determination of the total amount of sulpholactic acid in free spores the glucose–glutamate medium described by Warren (1968) was utilized, but with the replacement of $MgSO_4$ by $MgCl_2$.

Determination of the number of spores. Refractile spores were counted in the phase-contrast microscope and expressed as a percentage of the total number of cells. The number of heat-resistant spores was determined by heating samples of sporulating cultures at 80°C for 10min to kill vegetative cells and then plating suitable dilutions on nutrient agar.

Determination of 2,6-dipicolinic acid. Samples (50ml) from resuspension medium were autoclaved and then assayed for 2,6-dipicolinic acid by using the reagents of Janssen, Lund & Anderson (1958).

Measurement of protein. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Bacterial growth. This was determined from the extinction of cultures at 600nm (Mandelstam & Waites, 1968).

Preparation of spores. Cleaned preparations of spores were obtained by the method of Bonsen *et al.* (1969). The final suspension contained at least 95% refractile spores, the remainder consisted of phase-dark spores and less than 0.1% bacillary forms.

Enzyme assays and units. Alkaline phosphatase and glucose dehydrogenase were determined in bacterial samples taken from the resuspension medium described by Warren (1968). One unit of enzyme activity is defined as that amount catalysing the reaction of 1 nmol of substrate/min. Specific activities are calculated as units/ml of culture.

Preparation and characterization of L-*sulpholactic acid.* L-Sulpholactic acid [HO_2C–$CH(OH)$–CH_2–SO_3H] (L-2-carboxy-2-hydroxyethanesulphonic acid) was prepared from L-cysteic acid by a modification of the method of Dakin & Dudley (1914). L-Cysteic acid (5g) was dissolved in 2M-HCl (40ml) and cooled to 0°C. Ice-cold $NaNO_2$ (20ml, 15% w/v) was added dropwise over 60min. The solution was left for 2h at 0°C, at room temperature for 15h, and then repeatedly extracted with ethyl acetate. The combined extracts were evaporated *in vacuo* to give a pale-yellow solid which was recrystallized from ethanol–water to give a white solid (m.p.>250°C) (Found: C, 18.3; H, 3.3; S, 14.5. Calc. for $C_3H_6O_6S$: C, 21.2; H, 3.5; S, 18.8%). (The low values observed are due to the presence of an inorganic residue.) The compound was ninhydrin-negative, and was precipitated by $BaCl_2$ at pH6.5 but not at pH1. It was acidic and insoluble in ethanol. It did not react with 2,4-dinitrophenylhydrazine, suggesting the absence of a keto group. It migrated as a single acidic spot on high-voltage electrophoresis at the following pH values: 1.9, 3.5, 6.5 with $m_{sulphate}$, 0.5 at pH1.9 and 0.9 at pH3.5.

Assay of sulpholactic acid formed in cell suspensions. Cells in resuspension medium were incubated with 100μCi of [^{35}S]sulphate. Samples (normally 10 or 15ml) were centrifuged and the cell pellets resuspended in 5ml of water. The cells were then broken in a Braun homogenizer as described by Warren (1968) and mixed with 2ml of $HClO_4$ [0.75M in 50% (v/v) ethanol] and left for 1h. The precipitate was removed by centrifugation and the supernatant neutralized at 0°C with 2M-KOH and centrifuged. The supernatant was dried down and redissolved in water (0.2ml) containing carrier sulpholactic acid (100μg). Samples (0.1ml) were then taken for chromatography or electrophoresis. Separations by descending chromatography were carried out on Whatman no. 4 paper with either of the following solvents: (1) butan-1-ol–acetic acid–water (12:3:5, by vol.) (Smith, 1960); (2) propan-1-ol–M-formic acid (3:1, v/v) for 15h. Sulpholactic acid was detected by spraying the chromatograms with a solution of Cresol Red (0.04% in ethanol) which gave a red spot on a yellow background. Sulpholactic acid migrated with R_F0.05 in solvent (1) and 0.1 in solvent (2). High-voltage paper electrophoresis (Dreyer & Bynum, 1967) was used as an alternative to paper chromatography. Markers of [^{35}S]sulphate, unlabelled cysteic acid and methionine were included in all separations. Amino acids were detected by spraying with 0.2% ninhydrin in butan-1-ol. Labelling of sulpholactic acid was confirmed by radioautography of chromatograms for 1–3 weeks with Kodirex X-ray film. Radioactive spots were then eluted with water, dried down, redissolved in 0.1ml of water, and the total sample spotted on to glass-fibre paper and the radioactivity counted until at least 1000 counts were obtained.

RESULTS

Occurrence of sulpholactic acid in sporulating suspensions. Initial experiments were carried out to determine if *B. subtilis* 168 produced sulpholactic acid during sporulation, since Kadota & Uchida (1967) had presented evidence that in spores of *B. subtilis* Marburg (A.T.C.C. 6051) only cysteine and methionine were present as small-molecule sulphur compounds. A 50ml resuspension culture was treated at zero time with 50μCi of [^{35}S]sulphate, aerated for 7h and centrifuged. The extract from the whole-cell pellet was examined for radioactive sulpholactic acid as described in the Materials and Methods section. On electrophoresis the main ^{35}S-containing compound migrated towards the anode faster than cysteic acid, but slower than [^{35}S]sulphate, and coincided with authentic sulpholactic acid. Cysteic acid, methionine and trace amounts of other unidentified ^{35}S-labelled compounds were also detected, but the total radioactivity in all these was only 15% of that in sulpholactic acid. The ^{35}S-labelled compound presumed to be sulpholactic acid was eluted and, since it co-chromatographed with authentic sulpholactic acid in solvents (1) and (2), it was presumed to be identical with L-sulpholactic acid.

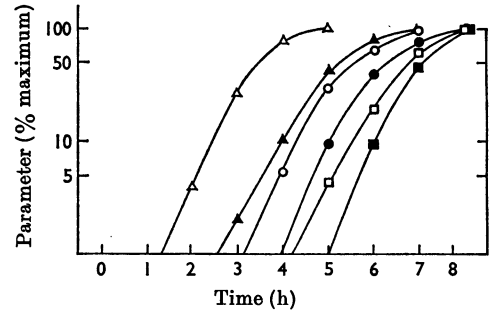

Fig. 1. Time-course of sulpholactic acid formation relative to the other biochemical events of sporulation. The values are expressed as a percentage of the maximum obtained for each parameter. Sulpholactic acid, alkaline phosphatase and refractility were measured on the same ^{35}S-labelled culture. Glucose dehydrogenase and 2,6-dipicolinic acid were assayed with samples from a separate 1 litre of unlabelled resuspension culture. Maximum values are given in parentheses. △, Alkaline phosphatase (14.2 units/mg of protein); ▲, glucose dehydrogenase (28.4 units/mg of protein); ○, sulpholactic acid (7200 c.p.m./10ml sample); ●, refractility (80% refractile spores); □, 2,6-dipicolinic acid (28μg/mg of protein); ■, heat resistance (80% heat-resistant forms/ml of culture).

Time of appearance of sulpholactic acid relative to the other events of sporulation. The synthesis of sulpholactic acid was followed by withdrawing, at hourly intervals, 10ml samples from 100ml of resuspension culture containing [^{35}S]sulphate (100μCi) and measuring the incorporation into sulpholactic acid. Alkaline phosphatase activity, refractility and heat resistance were measured on samples from this culture. Glucose dehydrogenase activity and 2,6-dipicolinic acid were determined on parallel non-labelled cultures since the quantity of cells required is five- or ten-fold greater than that required for sulpholactic acid determination. Synthesis of sulpholactic acid began between 3 and 4h after resuspension, and the rate paralleled that of other marker events of sporulation examined (see Fig. 1). It appeared 30min after glucose dehydrogenase and about 1h before the appearance of refractility. No difference in the rate of synthesis or time of appearance was observed in cultures from resuspension medium prepared with the normal concentration of sulphate salts. No radioactive sulpholactic acid could be detected in extracts of exponentially growing cells from a 50ml culture incubated with 250μCi of [^{35}S]sulphate for 3h.

Amount of sulpholactic acid in free spores. The amount of sulpholactic acid in free spores was measured by using the glucose–glutamate medium (Warren, 1968; see the Materials and Methods section) with magnesium chloride in place of magnesium sulphate, but containing sodium sulphate (1.875mM) to ensure constant specific radioactivity of [^{35}S]sulphate for labelling. A small inoculum of about 10^4 cells was added to 50ml of this medium and the culture aerated until 70% free spores were obtained after 24h. The spores and remaining vegetative cells were harvested and the spores separated as described (see the Materials and Methods section). Unlabelled spores were prepared from parallel unlabelled cultures to obtain an estimate of the dry weight of spores. The labelled spores were broken and extracted and the radioactivity incorporation into sulpholactic acid was measured. From this value and the calculated value of the specific radioactivity of sulphate the sulpholactic acid content was determined. The averages of two separate determinations gave sulpholactic acid as 1.7% of the dry weight of the spores. The results of two experiments are given in Table 1.

Effect of chloramphenicol on sulpholactic acid formation. To test whether the synthesis of sulpholactic acid required protein synthesis *de novo* or whether it was due to the activation of an existing enzyme, additions of chloramphenicol to a final concentration of 50μg/ml were made to 15ml samples taken from labelled sporulating cultures at hourly intervals after resuspension. This concentration of chloramphenicol is sufficient to inhibit protein synthesis in this species (Sterlini & Mandelstam, 1969). The samples were removed after 7h, assayed for incorporation of label into sulpholactic acid, and the amounts compared with those of control samples taken at the same time. The results of two separate experiments (Table 2) show that addition of chloramphenicol up to 3h after resuspension prevented any detectable formation of sulpholactic acid, and also any spore formation. Additions after this period allowed sulpholactic acid formation to take place but, since less was made than in the control cultures, it seemed that protein synthesis continuing over 2–3h was required for synthesis of sulpholactic acid to the normal concentration.

Synthesis of sulpholactic acid in asporogenous mutants. The asporogenous mutants blocked at various stages of sporulation that were examined by Waites *et al.* (1970) were assayed for their ability to synthesize sulpholactic acid. The mutants were grown in hydrolysed casein medium and then resuspended in ^{35}S-labelled medium. After 6h, two samples (15ml) were taken and assayed for incorporation of ^{35}S into sulpholactic acid. The value

Table 1. *Determination of the quantity of sulpholactic acid in free spores*

A small inoculum (approx. 10^4 cells) was added to 50ml of the modified glucose–glutamate medium containing sulphate with known specific radioactivity of ^{35}S (see the Materials and Methods section). The culture was grown for 24h until 70% free spores were obtained. The spores were purified and the incorporation of ^{35}S into sulpholactic acid was measured. Spores were similarly prepared from equal volumes of unlabelled medium to obtain an estimate of dry weight. From this value and the yield of sulpholactic acid the proportion of the latter in the spore was calculated.

	Expt. 1	Expt. 2
(*a*) Specific radioactivity of sulphate in the medium (c.p.m./μmol)	990	1008
(*b*) Total radioactivity of sulpholactic acid in sample after extraction from 50ml culture (c.p.m.)	1362	1134
(*c*) Concn. of sulpholactic acid (μmol/50ml culture) (*b*/*a*)	1.33 (=227μg)	1.05 (=179μg)
(*d*) Dry weight of spores/50ml culture	12.5mg	10.7mg
(*e*) Sulpholactic acid as % of dry weight of the spore (*c*/*d*)	1.82	1.67

Table 2. *Effect of chloramphenicol on sulpholactic acid synthesis*

A resuspension culture containing [^{35}S]sulphate was set up and, at hourly intervals, samples were removed and additions of chloramphenicol to a final concentration of 50 μg/ml were made. The cultures were incubated until 7 h from resuspension. Each was then examined for the number of refractile spores. The cells and spores were broken and the incorporation of label into sulpholactic acid was measured (see the Materials and Methods section). The 7 h value for sulpholactic acid taken as 100% was 8100 c.p.m./15 ml sample. The control culture contained 70% refractile spores after 7 h of incubation. The average of two such experiments is given.

Time of addition of chloramphenicol after resuspension (h)	Amount of sulpholactic acid (% of the control amount at 7 h from resuspension)	Refractile spores (% of control at 7 h)
0	0	0
1	0	0
2	0	0
3	0	0
4	4.5	0
5	24.0	3.0
6	52.0	33.0

Table 3. *Amounts of sulpholactic acid in asporogenous mutants*

Mutants and wild-type cells were grown and resuspended in ^{35}S-labelled medium. Duplicate samples were taken at 6 h and assayed for incorporation of ^{35}S into sulpholactic acid (see the Materials and Methods section). The results of two such experiments on each mutant are shown. The incorporation into sulpholactic acid in the 6 h sample from the wild-type was 4500 c.p.m./15 ml sample. This was taken as 100% and the results with asporogenous mutants are expressed in relation to this.

Mutant	Stage	Glucose dehydrogenase	Refractility	Expt. 1	Expt. 2
E22	0	—	—	0.9	0.6
T20	0	—	—	0.6	0.5
U42	0	—	—	0.2	0.7
N38	II	—	—	0.4	0.8
N25	II	—	—	0.7	0.6
NG82	II	—	—	0.5	0.8
E21	II	—	—	0.4	0.8
A24	II	—	—	0.6	0.3
NG13	III	—	—	0.7	0.4
E34	III	+	+	0.2	0.6
A8	IV	+	+	12.4	15.0
E33	IV	+	+	10.1	8.5
E31	IV	+	+	24.2	18.6
A23	IV	+	+	34.7	31.7

was compared with that found in the wild-type which was also sampled 6 h after resuspension. The results of two such experiments are shown in Table 3. No significant amounts of sulpholactic acid were observed in any of the mutants blocked before stage IV. All the mutants blocked at stage IV produced sulpholactic acid, but in all of them the amounts of sulpholactic acid were substantially less than those found in experiments with the wild-type.

Effect of sulpholactic acid on sporulation of wild-type and on asporogenous mutants. The following experiments were carried out to determine whether sulpholactic acid had any obvious effect on the sporulation of wild-type and whether it would restore the ability to sporulate in any of the mutants. Sulpholactic acid was added to the resuspension medium at final concentrations of 25, 50, 100, 200 and 500 μg/ml. Exponentially growing cells were then resuspended and allowed to sporulate. The numbers of refractile spores and heat-resistant spores were determined hourly up to 7 h after resuspension. No differences in the rate or amount of sporulation could be detected.

Sulpholactic acid was incorporated into nutrient agar plates at a concentration of 500 μg/ml and the asporogenous mutants were streaked on to this medium and incubated at 37°C for 3 days. Control plates were also prepared by omitting sulpholactic

acid. The addition of sulpholactic acid did not cause the asporogenous mutants to produce refractile or heat-resistant spores.

DISCUSSION

In the resuspension medium used for these experiments glucose dehydrogenase activity, a marker event in sporulation of *B. subtilis*, can first be detected at about 3h after the initiation of sporulation and refractility at 4h (Waites *et al.* 1970). The formation of sulpholactic acid occurred some 30min after glucose dehydrogenase and about 1h before the development of refractility. Since sporulation is asynchronous in this system, events are spread over a 2–3h period and can best be separated by the use of semi-logarithmic plots as shown in Fig. 1. This timing of sulpholactic acid synthesis corresponds to the end of stage III and the beginning of stage IV, a point where spore protoplast formation is complete and cortex formation is commencing (Dawes, Kay & Mandelstam, 1969). The amount of sulpholactic acid, 1.7% of the spore dry weight, was less than that found by Bonsen *et al.* (1969). This may be due to the use of a different strain of *B. subtilis*.

Sulpholactic acid was not found in asporogenous mutants blocked in earlier stages, i.e. before stage IV in the sporulation sequence. The mutants that were able to produce significant amounts of sulpholactic acid also showed partial refractility and the presence of glucose dehydrogenase. In none of these mutants did the amount of sulpholactic acid approach that of the wild-type although it reached 34% of wild-type concentration in mutant A23 (see Table 3). The decreased amounts compared with wild-type concentration are proportionally similar to those recorded for glucose dehydrogenase in these mutants (Waites *et al.* 1970). The association of sulpholactic acid with stage IV was further confirmed by its absence from mutant E34 which has been designated as a stage III mutant on the basis of its morphology. The fact that production of sulpholactic acid is correlated with a particular stage in the sporulation process suggests that it may be specifically concerned with that process and is not produced as a consequence of the 'shift-down' to a minimal medium. Nevertheless the function of sulpholactic acid in sporulation of the Marburg strain remains unknown. To determine what it is would require the isolation of mutants blocked specifically in sulpholactic acid synthesis. These could then be examined for their ability to sporulate and for the altered properties of such spores as they might produce. Sulpholactic acid does not appear to be present in the spores of some other strains of *B. subtilis* (Kadota & Uchida, 1967) or in other *Bacilli* (Bonsen *et al.* 1969). It may thus be similar to the pigment formed in sporulation, which appears to be a secondary product of sporulation (Mandelstam, 1969). Even so, the correlation between its appearance and the stage of sporulation reached by wild-type or mutant cultures of *B. subtilis* 168 makes it a useful event to study.

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