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Actinomycin Biosynthesis by Protoplasts Derived from *Streptomyces parvulus*

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Conditions are described for the formation of protoplasts from *Streptomyces parvulus* that are able to synthesize actinomycin D de novo. Antibiotic synthesis by protoplasts, in contrast to that by mycelium, was sensitive to inhibition by actinomycin D and to a decrease in sucrose concentration. On the other hand, synthesis by mycelium was much more sensitive to inhibition by amino acid analogs (D-valine, *cis*-3-methylproline, and α-methyl-DL-tryptophan). In addition, the uptake of amino acids (L-methionine, sarcosine, and L- and D-valine) by protoplasts was significantly lower than that by mycelium. The advantages and limitations of using protoplasts for studying in vivo actinomycin synthesis are discussed.

The mechanism of nonribosomal, protein thiotemplate synthesis of peptide antibiotics, first established for the biosynthesis of gramicidin S and tyrocidine (26, 27), appears also to be the mechanism by which bacitracin (11, 33), edeine (25), and linear gramicidin (1, 3) are formed. In the case of a number of other peptide antibiotics, the use of inhibitors in vivo has shown that the mechanisms of protein and antibiotic syntheses differ (21), but further insight has been precluded by the difficulty in obtaining cell-free systems that will produce the antibiotics. For this reason, studies relating to the biosynthesis of actinomycin, for example, have invariably been carried out with intact mycelium (20).

Cell extracts prepared by mechanical disruption are often inactive in antibiotic synthesis; however, effective preparations have been obtained in a number of cases by incubation of intact cells with lysozyme in a hypotonic buffer (e.g., 1, 3, 12, 13, 25, 30, 34). In the case of *Bacillus licheniformis*, cells were treated first with lysozyme in hypertonic buffer to produce protoplasts capable of antibiotic synthesis (39); subsequent osmotic disruption gave rise to a cell-free system that synthesized bacitracin (18). Recently, Okanishi et al. (32) described the formation of protoplasts and their reversion to filamentous forms in studies with *Streptomyces griseus* and *Streptomyces venezuelae*; however, no attempt was made to examine the protoplast systems for antibiotic production. By contrast, Keller and Kleinkauf (personal communication) have demonstrated that actinomycin synthesis occurs in protoplasts derived from *Streptomyces antibioticus*. This organism produces relatively little actinomycin (70 to 100 μg/ml), and the mixture elaborated consists of two major (actinomycins IV and V) and several minor (actinomycins I, II, and III) components (20). *Streptomyces parvulus*, on the other hand, synthesizes only one actinomycin to any significant extent (IV, >95%) (31), in larger amounts (600 to 700 μg/ml) (unpublished data). In this report, we describe the formation and properties of *Streptomyces parvulus* protoplasts that synthesize actinomycin de novo.

MATERIALS AND METHODS

Organism and conditions of cultivation. *S. parvulus* ATCC 29651 was maintained and cultivated as previously described for the parent strain (ATCC 12434), with the glutamic acid-histidine-fructose-mineral salts (GHF) medium being used for maximum antibiotic titers (40).

Preparation of protoplasts. The protoplast preparation method used was a modification of that described by Keller and Kleinkauf (personal communication). Mycelium was harvested from GHF medium after 36 to 48 h of growth (except where stated) by suction filtration on a Büchner funnel and washed with 10% (wt/vol) sucrose. Five milliliters of protoplast buffer [25 mM *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid-25 mM $CaCl_2O$-10 mM $MgCl_2 \cdot 6H_2O$-10% (wt/vol)-sucrose-1% (wt/vol) fructose, pH 7.6, filtered through a 0.45-μm membrane filter (HAWP; Millipore Corp., Bedford, Mass.) before use] was added per gram (wet weight) of mycelium, and, after dispersion of the mycelium, lysozyme was added (2.5 mg/ml of

buffer). The mixture was then gently shaken at 100 rpm and 30°C in a G-24 environmental incubator shaker (New Brunswick Scientific Co., New Brunswick, N. J.).

After incubation with lysozyme for 2 h, the mycelium was removed by filtration (first through loosely packed and then through tightly packed glass wool). The protoplasts in the filtrate were sedimented by acceleration in a centrifuge to 10,000 × *g* at 20°C followed by immediate deceleration. The pellet was washed by suspending it in the original volume of fresh protoplast buffer, and the protoplasts were again sedimented in the same way. The pelleted material was then resuspended in 2 to 3 ml of protoplast buffer and filtered once again through tightly packed glass wool. This suspension was then diluted to give an E_{650} of 8.0 in a Zeiss PM-Q II spectrophotometer. The number of protoplasts in such a preparation, as determined in a Petroff-Hausser counting chamber, was variable, but the protein content was relatively constant (see Results).

Microscopy. Protoplasts were observed with a Nikon (model LBR-Ke) microscope with phase-contrast optics. The number of protoplasts in a given suspension was evaluated by diluting an aliquot in protoplast buffer to give an E_{650} reading of approximately 0.15; protoplasts were then counted in a Petroff-Hausser counting chamber.

Estimation of protoplast formation. Protoplast formation as a function of time was determined by withdrawing 0.5-ml portions of the mixture of mycelium and lysozyme and mixing each with 2 ml of protoplast buffer. Mycelium was removed by gravity filtration through glass wool, and the E_{650} of the filtrate was measured against a reference cell containing protoplast buffer.

Actinomycin synthesis by protoplasts. Incubation mixtures [200 μl final volume, containing 5 μmol of *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid, 5 μmol of $CaCl_2$, 2 μmol of $MgCl_2$, 20 mg of sucrose, 1.0 mg of D-fructose, and about 330 μg of protein as protoplasts] were shaken in test tubes (15 by 1.5 cm) held vertically in racks in a Dubnoff metabolic shaking incubator operating at 30°C and 120 cycle/min. For routine incubations, either 0.5 μCi of L-[*methyl*-^{14}C]methionine or 1.0 μCi of L-[*U*-^{14}C-]valine was used as the substrate, and 100 μl of protoplast suspension was added to start the reaction. Reactions were terminated after 60 min (except where stated otherwise) by addition of 1.8 ml of ice-cold deionized water. Five microliters of actinomycin IV solution (1 mg/ml) was added as the carrier, followed by 0.1 ml of 2 M tris(hydroxymethyl)aminoethane-hydrochloride, pH 8.0, to maintain pH control during extraction. The above mixture was extracted with 4.0 ml of ethyl acetate, and, after centrifugation, the organic phase was washed with 3 ml of 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 8.0. A 0.4-ml aliquot of the washed organic phase was used for radioactive measurement in 10 ml of Bray liquid scintillation fluid.

Actinomycin synthesis by mycelium. Procedure A. Incubations were effected in 50-ml conical flasks shaken at 120 cycles/min and 30°C in a Dubnoff metabolic shaking incubator. Incubations contained 4.0 ml of culture (grown in GHF medium for 36 to 48 h) and 50 μl of a solution of radioactive amino acid (containing 0.05 μCi and 0.05 μmol), and the volume was made up to 5.0 ml with water or with an aqueous solution of a test compound (e.g., inhibitors). Reactions were stopped after 15 min by filtration of the mixture through glass wool into a test tube held in ice. Two milliliters of the filtrate was then extracted with 4.0 ml of ethyl acetate, and, after low-speed centrifugation, the organic phase was washed with 3 ml of 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 8.0. Two milliliters of the washed organic phase was used for radioactive measurement.

Procedure B. When a direct comparison was made in experiments with mycelium versus protoplasts, the mycelium was harvested and treated in a manner similar to that described above for the protoplast preparation, except that lysozyme was omitted during the 2-h incubation. The mycelium was then harvested by suction filtration on a Büchner funnel and washed with the protoplast buffer. The mycelium was then suspended in protoplast buffer (5 ml/100 ml of original culture volume), and 1.0 ml of this preparation was used in the experiments. The final composition of the 5-ml incubation mixtures was identical to that used with protoplasts, except for the amount of radiolabeled substrate (0.05 μmol, 0.05 μCi). Incubations were for 30 min; extractions were carried out as in procedure A.

Inhibition of actinomycin synthesis: mycelium versus protoplasts. The effect of inhibitors (e.g., α-methyl-DL-tryptophan, D-valine, *cis*-3-methyl-DL-proline, and actinomycin D) on actinomycin synthesis was determined by the assay procedures given above, except that cells were preincubated with the inhibitor for 10 min under the assay conditions before addition of the radioisotope.

Estimation of protein. Protein was assayed by the method of Lowry et al. (28), with bovine serum albumin as the standard. Mycelial protein was prepared by precipitating an aliquot of the culture with an equal volume of 10% (wt/vol) trichloroacetic acid. The precipitate was washed three times with ice-cold 5% trichloroacetic acid and then heated (100°C, 30 min) with 5% trichloroacetic acid. The insoluble precipitate was heated at 100°C for 30 min in 0.5 M KOH; undissolved material was removed by centrifugation, and aliquots of the supernatant were assayed.

To determine protoplast protein, 1 volume of the protoplast preparation was heated (100°C, 30 min) with 9 volumes of 0.5 M KOH. After mixing with 40 volumes of deionized water, insoluble material was sedimented by centrifugation, and aliquots of the supernatant were assayed. No significant difference in protein content was noted when protoplast protein was treated as noted above or precipitated initially with trichloroacetic acid before solubilization. Assays of standard bovine serum albumin were unaffected by protoplast buffer when used at the same concentration as present in samples.

Chromatographic and electrophoretic procedures. Products extracted into ethyl acetate at pH 8.0 from various incubations were dried under vacuum at 40 to 50°C with a Büchi Rotavapor or a Buchler Evapomix and redissolved in a small volume of methanol. Samples were applied to silica gel thin-layer chromatography (TLC) plates (60 F-254; Merck & Co., Inc., Rahway, N. J.), which were developed with ethyl acetate-methanol-water (20:1:1, by volume).

Separation of amino acids in actinomycin hydrolysates or aqueous filtrates (uptake experiments with protoplasts) was accomplished by electrophoresis with a high-voltage Electrophorator model D (Gilson Medical Electronis, Inc., Middleton, Wis.) on Whatman 3 MM paper and 4% formate buffer, pH 1.9. Amino acids in actinomycin hydrolysates were separated at 160 mA for 3.5 h; filtrates from protoplast experiments were developed at 50 mA for 0.5 h followed by 200 mA for 3 h. After location of radioactive regions, amino acids were visualized with 0.2% ninhydrin in acetone.

Radioactive measurements. Radioactive measurements of liquid samples were effected in a refrigerated Nuclear Chicago Mark I spectrometer with 10 ml of Bray (5) scintillation fluid (70% efficiency). For most experiments, quenching due to actinomycin was insignificant and constant. However, when the effect of actinomycin D on antibiotic synthesis was studied, significant quenching was observed at higher concentrations of the drug. Under these conditions, appropriate corrections were made to eliminate this effect. ^{14}C-labeled metabolites (e.g., actinomycin D) located on TLC plates were detected by radioautography, using DuPont Cronex 4 daylight pack or Kodak SB-5 X-ray film. High-voltage electrophoresis paper strips were examined with a Packard radiochromatogram scanner (model 7201; Packard Instrument Co., Inc., Downers Grove, Ill.).

Distribution of radiolabeled precursors in actinomycin D. The region on a TLC plate containing actinomycin D (^{14}C-labeled plus ^{12}C-carrier actinomycin) was scraped off the plate and extracted twice with 5 ml of methanol. The combined extracts were dried *in vacuo*, and the actinomycin was hydrolyzed in 6 N HCl in a Teflon-lined, screw-capped test tube. Hydrolysates were evaporated to dryness at 60 to 70°C, taken up in water and dried three times to remove HCl. Hydrolysate amino acids were dissolved in a small volume of 80% aqueous methanol and applied to Whatman 3 MM paper along with approximately 0.2 μmol of the appropriate amino acids (proline, sarcosine, threonine, valine, and *N*-methylvaline). Separation was then accomplished by high-voltage electrophoresis.

Uptake of amino acids by protoplasts and mycelium. Uptake of amino acids was measured by the disappearance of the ^{14}C-amino acid from the medium and incubation mixtures.

For studies with mycelium, procedure A (see above) was used, and, after filtration as described, 2 ml of the culture filtrate was employed. For experiments with protoplasts, 0.2-ml incubation mixtures were diluted with 1.8 ml of protoplast buffer at the appropriate times, and the protoplasts were removed by filtration *in vacuo*, using a membrane filter apparatus (Millipore HAWP, 0.45 μm); 1 ml of filtrate was then mixed with 1 ml of deionized water. In either case, the solution (2 ml) was adjusted to pH 2.0 to 2.5 before extraction twice with ethyl acetate (4 ml). Aliquots of the aqueous layer were then used for radioactivity measurement.

In studies with mycelium, the amount of amino acid remaining in the medium was calculated from the ^{14}C determination and the known specific activity. In studies with protoplasts, aliquots of the aqueous phase were mixed with the appropriate ^{12}C-amino acid (sarcosine, methionine, or valine) and developed by high-voltage electrophoresis. After drying, strips were cut from the electropherograms and examined with the radiochromatogram scanner to determine the location and percentage of radiolabel in the compounds present. With [^{14}C]methionine or L-[^{14}C]valine as substrates, nonextractable radioactive metabolites were produced during the incubations. Total counts in the aqueous phase were corrected by using the percentage values obtained to give counts due to the added amino acid, and the amount (in nanomoles) was then calculated from the specific activity.

Radioisotopes. L-[*methyl*-^{14}C]methionine, 48.9 mCi/mmol; L-[*U*-^{14}C],valine, 251 mCi/mmol; L-[*U*-^{14}C]threonine, 203 mCi/mmol; [*methyl*-^{14}C]sarcosine, 3.86 mCi/mmol; L-[*U*-^{14}C]lysine, 273 mCi/mmol; L-[*U*-^{14}C]aspartic acid, 192 mCi/mmol; L-[side chain-3-^{14}C]tryptophan, 56.8 mCi/mmol; and L-[*U*-^{14}C]tryptophan, 566 mCi/mmol were purchased from New England Nuclear Corp., Boston, Mass. DL-[benzene ring-*U*-^{14}C]tryptophan, 60 mCi/mmol; L-[*U*-^{14}C]proline, 275 mCi/mmol; and [*U*-^{14}C]glycine, 107 mCi/mmol were obtained from Amersham/Searle, Des Plaines, Ill.

Chemicals and reagents. Lysozyme, 9,000 U/mg (Worthington Biochemicals Corp., Freehold, N.J.), *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (Sigma Chemical Co., St. Louis, Mo.), sucrose (J. T. Baker Chemical Co., Phillipsburg, N.J.), and bovine serum albumin fraction V (Sigma) were reagent grade. Amino acids were obtained from commercial sources. α-Methyl-DL-tryptophan was kindly supplied by Merck Sharp & Dohme Research Laboratories (Rahway, N.J.), and *cis*-3-methyl-DL-proline was a gift from A. Mauger, Washington Hospital Center (Washington, D.C.).

RESULTS

Formation of and actinomycin synthesis by protoplasts as a function of age of *S. parvulus* cultures. Mycelium harvested at various times after inoculation into GHF medium was incubated with lysozyme, and the rate and extent of protoplast formation were observed. It can be seen (Fig. 1) that the amount of protoplasts produced after 2 h was greater when the mycelium was physiologically young (24 to 48 h) and that this property declined with aging of the culture. A similar development of resis-

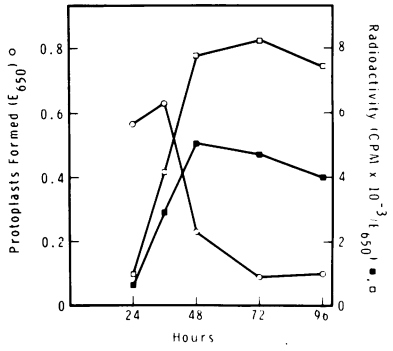

FIG. 1. *Formation of and actinomycin synthesis by protoplasts prepared from S. parvulus mycelium at different ages. Mycelium at different ages, cultivated in GHF medium, was converted to protoplasts, and the amount of protoplasts formed (○) was estimated spectrophotometrically as described in the text. Actinomycin-synthesizing activity was measured by incorporation of radiolabel from [methyl-^{14}C]methionine into ethyl acetate-extractable material after incubation with protoplasts for 60 (■) or 120 (□) min.*

tance to lysozyme with increase in culture age was noted by Keller and Kleinkauf (personal communication). The yield of protoplasts was essentially complete after 2 h with lysozyme in all cases studied, except when mycelium harvested at 24 h was used. In this particular instance, the initial rate of protoplast formation was slower than that observed with mycelium taken from 36- and 48-h cultures; however, it continued during incubations up to 3.5 h, at which time the yield of protoplasts was about 2.5-fold greater than that at 2 h.

Microscopic examination of the protoplast suspensions failed to reveal any evidence of contamination by mycelial fragments; however, in preparations harvested at 48 h or later, some degree of contamination by spores was visible (<5%).

Protoplasts harvested after 2 h with lysozyme were washed and resuspended in protoplast buffer as noted in Materials and Methods. The number of protoplasts per milliliter per unit of E_{650}, as measured with the Petroff-Hausser chamber, was found to vary between 0.16×10^9 and 2.0×10^9. Differences in the sizes of the protoplasts in different preparations probably account for the observed variation. By contrast, the protein content per milliliter per unit of E_{650} was fairly constant (410 ± 50 μg).

The ability of the protoplasts produced from mycelium at different ages to incorporate methyl equivalents (from [*methyl*-^{14}C]methionine) into actinomycin was also determined (Fig. 1). Protoplasts from 24-h-old mycelium synthesized little radiolabeled actinomycin. This was not surprising, since antibiotic synthesis at 24 h was negligible (<1.0 μg/ml of culture). However, the actinomycin-synthesizing ability of the protoplasts increased with mycelial age (at 36 and 48 h) and then remained fairly constant up to 96 h.

Further experiments were undertaken with protoplasts derived from mycelium harvested between 36 and 48 h, as this constituted the most suitable period for obtaining the maximum amount of protoplasts with a high level of antibiotic-synthesizing activity.

Effect of different ^{14}C-amino acids on actinomycin synthesis by protoplasts. Protoplasts were incubated with 1.0 μCi of various ^{14}C-amino acids, and the amount of radiolabel incorporated (ethyl acetate-extractable material) was determined (Table 1). All known constituent amino acid precursors of actinomycin D (23) (methionine, tryptophan, proline, valine, threonine, and glycine) were incorporated to some extent, varying between 8- and 86-pmol equivalents of radioactive actinomycin after 1 h of incubation and between 13.2- and 170-pmol equivalents after 2 h. These differences in incorporation may reflect differences in the intracellular pool concentrations of the various amino acids, in the rates of entry of the amino acids into the protoplasts, and in the metabolic requirements of the organism for specific amino acid substrates. Sarcosine, which can be incorporated into actinomycin by mycelium of *S. antibioticus* (7, 8) and *S. parvulus* (see below), and lysine, which is not an actinomycin precursor, were not incorporated. However, L-aspartic acid and D-valine were incorporated to a slight extent.

The ethyl acetate-extractable material was analyzed by TLC and radioautography (Fig. 2). With glycine, methionine, threonine, proline, and L-valine as radiolabeled precursors, only one major band of radioactivity was present, which migrated to the same position as the carrier actinomycin D (R_f = 0.42) added before extraction. However, with [benzene ring-*U*-^{14}C]tryptophan, radioactivity was found not only associated with actinomycin D, but also with several other bands, at R_f values of 0.38, 0.54, 0.84, and 0.98. Radiolabel from aspartic acid was not present in actinomycin D, but appeared in a single band that migrated with the solvent front, and the amount of radioactivity incorporated from D-[^{14}C]valine was too weak to detect (not shown).

Actinomycin D from incubations of protoplasts with radiolabeled precursors was ex-

TABLE 1. *Incorporation of radioactivity into ethyl acetate-extractable material by protoplasts from S. parvulus*[a]

Radiolabeled precursor	Radioactivity incorporated			
	1-h incubation		2-h incubation	
	cpm extracted[b]	pmol equivalents of actinomycin D[c]	cpm extracted[b]	pmol equivalents of actinomycin D[c]
L-[*methyl*-^{14}C]methionine	29,910	63.7	51,290	109.2
DL-[benzene ring-*U*-^{14}C]tryptophan	16,510	86.0	32,660	170.0
L-[*U*-^{14}C]proline	18,980	21.6	29,080	33.1
L-[*U*-^{14}C]valine	12,790	8.0	21,210	13.2
L-[*U*-^{14}C]threonine	8,390	12.9	16,890	26.0
[*U*-^{14}C]glycine	4,450	13.0	11,810	34.5
[*methyl*-^{14}C]sarcosine	0		0	
L-[*U*-^{14}C]lysine	0		0	
L-[*U*-^{14}C]aspartic acid	1,120		2,370	
D-[1-^{14}C]valine	150		600	

[a] Protoplasts were incubated with 1.0 μCi of each radiolabeled amino acid for 1 and 2 h.

[b] Values for counts per minute extracted are given as the differences between counts extracted after incubation for the times shown and counts extracted at zero time.

[c] Picomole equivalents of actinomycin D are calculated by assuming that each amino acid contributes two residues per actinomycin D molecule, except valine (four) and methionine (six). Picomoles of amino acid incorporated are calculated from the specific activity of the ^{14}C-labeled precursor and the counting efficiency (70%).

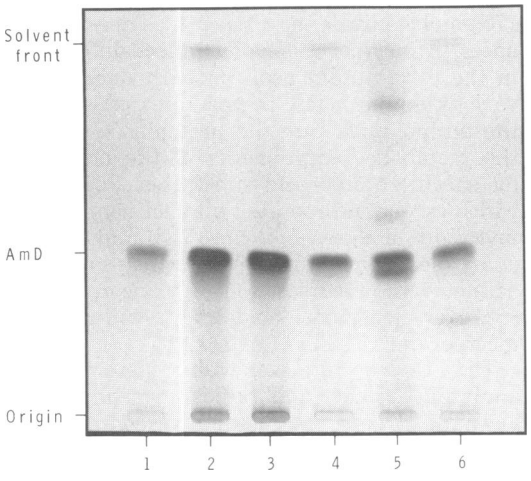

FIG. 2. *TLC and radioautography of ethyl acetate-extractable material from incubations of protoplasts with various ^{14}C-amino acids. Protoplasts were incubated for 1 h with 1.0 μCi of (1) [U-^{14}C]glycine, (2) L-[methyl-^{14}C]methionine, (3) L-[U-^{14}C]proline, (4) L-[U-^{14}C]threonine, (5) DL-[benzene ring-U-^{14}C]tryptophan, and (6) L-[U-^{14}C]valine. Ethyl acetate-extractable material was chromatographed with the ethyl acetate–methanol–water solvent system described in the text. Am D, Actinomycin D.*

tracted from TLC plates and hydrolyzed, and the hydrolysate amino acids were separated by high-voltage electrophoresis. As expected from studies with mycelium (23), radioactivity incorporated into actinomycin D from methionine was associated with *N*-methylvaline and sarcosine, whereas that derived from L-valine was present only in valine and *N*-methylvaline. Similarly, [^{14}C]threonine and [^{14}C]proline labeled actinomycin D only at the threonine and proline sites, respectively. ^{14}C radiolabel from glycine was located mainly in sarcosine (94%) and also in *N*-methylvaline (6%), in agreement with previous studies with intact mycelium (23). No significant radioactivity was present in the hydrolysate amino acids after analysis of the [benzene ring-*U*-^{14}C]tryptophan experiment. Previous investigations have shown that tryptophan labeled in the benzene ring is incorporated solely into the actinomycin chromophore, which is destroyed upon vigorous acid hydrolysis (38).

Utilization of different radiolabeled tryptophans as precursors. Incorporation of radiolabel from tryptophan into ethyl acetate-extractable metabolites was further investigated by using various labeled tryptophans. The addition of [side chain-3-^{14}C]-, [*U*-^{14}C]-, or [benzene ring-labeled *U*-^{14}C]tryptophan resulted in the synthesis of labeled, ethyl acetate-extractable metabolites. Analysis of these samples by TLC (Fig. 3) revealed that labeled actinomycin D was synthesized only in incubations with [benzene ring-*U*-^{14}C]- and [*U*-^{14}C]tryptophan, but not with [side chain-3-^{14}C]tryptophan. With the former two labeled tryptophans, a very significant amount of radioactivity was associated

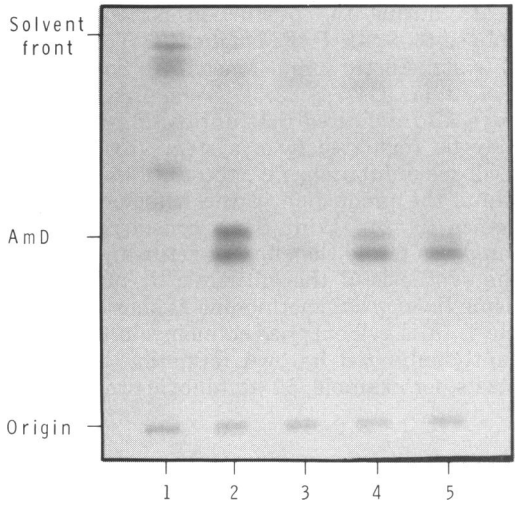

FIG. 3. *TLC and radioautography of ethyl acetate-extractable material from incubations of protoplasts with various ^{14}C-labeled tryptophans. Protoplasts were incubated for 1 h with 1.0 μCi of [^{14}C]tryptophan. Incubations contained (1) L-[side chain-3-^{14}C]tryptophan, (2) L-[U-^{14}C]tryptophan, (3) DL-[benzene ring-U-^{14}C]tryptophan plus 0.4 μmol of unlabeled L-tryptophan, (4) DL-[benzene ring-U-^{14}C]tryptophan, and (5) DL-[benzene ring-U-^{14}C]tryptophan plus 0.4 μmol of unlabeled D-tryptophan. Chromatography was carried out as described in Fig. 2. AmD, Actinomycin D.*

with an unknown metabolite with an R_f of 0.38. A 50-fold excess of unlabeled L-tryptophan (but not unlabeled D-tryptophan) decreased the conversion of radiolabel from DL-[benzene ring-U-^{14}C]tryptophan into both actinomycin (R_f = 0.42) and the metabolite with an R_f 0.38. These results suggest that both actinomycin D and the compound with an R_f of 0.38 contain the benzene ring moiety derived from L-tryptophan (but not D-tryptophan) by a sequence of reactions in which the side chain is removed.

Effect of storage of mycelium on formation of protoplasts and their ability to synthesize actinomycin. Mycelium from 500-ml volumes of cultures grown in GHF medium was harvested at 43 h, washed with 10% sucrose, and stored at either 4 or −20°C. It was noted that formation of protoplasts from stored mycelium was somewhat variable (±50%) in relation to that of freshly harvested mycelium. With protoplasts formed from mycelium stored at −20°C, significant actinomycin synthesis from [^{14}C]methionine or [^{14}C]valine (about 60% of that obtained with freshly harvested mycelium) was found, even after 4 weeks' storage. However, with protoplasts derived from mycelium stored at 4°C, only about 10% of the original activity remained after 1 week of storage. Because of the variability observed in both protoplast formation and biosynthetic activity of the protoplasts obtained from stored mycelium, further experiments were performed with freshly harvested mycelium.

Effect of static versus shaken incubations on actinomycin synthesis by protoplasts. Keller and Kleinkauf used static conditions for actinomycin synthesis by protoplasts of *S. antibioticus* and demonstrated that oxygen was required for antibiotic synthesis. Therefore, we compared the effects of static versus shaking conditions of incubation (Fig. 4). In both instances, the reaction was essentially completed in 120 to 150 min. However, with shaking, significantly more radioactivity was incorporated into the ethyl acetate fraction, especially during the period between 60 and 120 min (>70 to 120%). Analysis of the radioactivity in these fractions by TLC revealed that the major component in extracts of the static incubations was not actinomycin D but a compound that migrated with the solvent front. By contrast, in the extract derived from shaken incubations, the major component was actinomycin D.

Influence of various energy sources on actinomycin synthesis by protoplasts. The sigmoid shape of the time course of [*methyl*-^{14}C]methionine incorporation into actinomycin by protoplasts in shaken incubations (Fig. 4) is different from that seen with mycelial preparations (Fig. 5). This suggested the possibility of a decreased rate of transport of methionine as well as other actinomycin precursors into the protoplasts due to membrane defects arising during protoplast formation or to a need for supplemental energy or carbon sources. Therefore, various electron donors and alternative carbon sources were added to incubations to

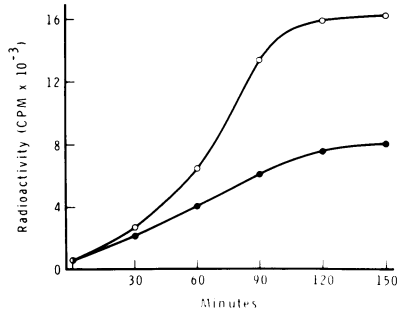

FIG. 4. *Effect of static versus shaken incubations on actinomycin synthesis by protoplasts. Incubations of protoplasts with [methyl-^{14}C]methionine were carried out under static (●) or shaking (○) conditions.*

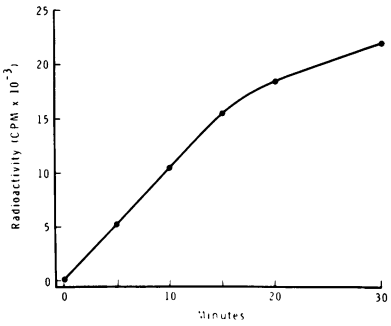

FIG. 5. *Synthesis of radiolabeled actinomycin from [methyl-^{14}C]methionine by S. parvulus mycelium as a function of time.*

determine whether the initial rate of synthesis could be enhanced. Addition of glucose, galactose, glucose-6-phosphate, L-lactate, D-lactate, succinate, phosphoenolpyruvate, DL-α-glycerophosphate, or DL-α-hydroxybutyrate (all at final concentrations of 20 mM) had little effect on the activity of protoplasts (101 to 109% of the control value); addition of ascorbate (20 mM) with phenazine methosulfate (0.1 mM) reduced activity markedly (84% inhibition).

By contrast, fructose was shown (Table 2) to be required for maximum antibiotic formation by protoplasts. Omission of fructose did not alter the yield of protoplasts, but the ability of the protoplasts to produce actinomycin was decreased 45%; however, this effect could be partially reversed by addition of fructose to the assay mixture. Therefore, protoplasts were always prepared in buffer containing fructose, and the latter served as the energy source for protoplasts in the ^{14}C-radiolabeled incubations.

Distinguishing features of protoplast versus mycelial systems. It was necessary to demonstrate that the actinomycin-synthesizing activity measured in our preparations was due to protoplasts rather than to a small, visually undetectable amount of mycelial fragments that may have passed through the glass wool filters. A number of experiments were carried out to eliminate this possibility.

(i) Osmotic stability. First, we examined the effect of decreasing the concentration of sucrose in the incubations (Table 3). With protoplasts, the activity was severely diminished by decreasing the concentration of sucrose, whereas with mycelium, either with or without previous exposure to 10% sucrose, synthesizing activity was fairly constant, regardless of the sucrose concentration.

(ii) Effect of actinomycin D. Several reports have noted that microorganisms become resistant to their own antibiotics, thus avoiding cell death during the production period, or idiophase (6, 9, 42; P. F. Hughes, T. Troost, and E. Katz, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, O47, p. 253). Some investigators have also indicated that antibiotic production may be regulated by a system analogous to feedback inhibition; i.e., the antibiotic per se limits the production of the homologous substance (9, 24). With these considerations in mind, we tested the effect of actinomycin D on the synthesis of the antibiotic by protoplasts using [*methyl*-^{14}C]methionine as substrate (Table 4). It is evident that actinomycin D significantly inhibited its own formation by protoplasts; for example, 50 μg/ml or more resulted

TABLE 2. *Effect of fructose as energy source on actinomycin synthesis by protoplasts*[a]

Protoplast prepn	Incubation with [*methyl*-^{14}C] methionine	cpm extracted/ incubation	% of control
With fructose (1.0%)	With fructose (0.5%)	59,970	100
Without fructose	Without fructose	32,990	55
Without fructose	With fructose (0.5%)	41,980	70

[a] Protoplasts were prepared in fructose-containing buffer as described in the text, or fructose was omitted from the buffer used during incubation with lysozyme and for washing the protoplasts. Activity of protoplasts was determined with L-[*methyl*-^{14}C]methionine (0.5 μCi) as substrate; incubation was for 1 h.

TABLE 3. *Effect of sucrose concentration on actinomycin synthesis by protoplasts and mycelium*[a]

Sucrose concn (%)	cpm extracted/incubation		
	Protoplasts	Mycelium	
		Procedure A	Procedure B
0		15,825	
2	558	16,320	8,395
4	791	17,365	9,360
6	728	16,545	9,100
8	3,227	17,665	8,830
10	8,868	16,960	9,075

[a] The effect of sucrose concentration on actinomycin synthesis was determined, using the assays described in the text with L-[*methyl*-^{14}C]methionine as substrate. The assay for mycelium in procedure A involved adding sucrose to mycelium at the same time as the radiolabel, whereas with procedure B, mycelium was incubated in buffer containing 10% sucrose for 2 h before assay (for further details, see the text). To enable the sucrose concentration in the protoplast preparation to be diluted down to 2%, 40 μl of a preparation with an E_{650} value of 20 replaced the usual 100 μl of a preparation with an E_{650} of 8.0.

TABLE 4. *Effect of actinomycin D on actinomycin synthesis by protoplasts and mycelium*[a]

Actinomycin D concn (μg/ml)	cpm extracted/incubation			
	Protoplasts	Mycelium		
		Procedure A	Procedure B	
			Expt 1	Expt 2
0	22,550	5,125	7,815	8,545
5	17,240			
10	12,250		9,330	10,355
20	9,530	4,565	9,885	10,895
50	5,840	4,895	10,155	11,205
100	6,140	5,460		11,215

[a] Mycelium was assayed without (procedure A) or with (procedure B) a prior 2-h incubation in protoplast buffer. Mycelium and protoplasts were incubated with actinomycin D at the concentrations given for 10 min before addition of L-[*methyl*-^{14}C]-methionine.

in almost an 80% inhibition. The ^{14}C-radiolabeled material in the ethyl acetate extract was chromatographed on silica gel TLC plates, and a radioautograph was prepared (Fig. 6). From an inspection of these results, it is clear that the inhibition of synthesis may have been far greater than 80%, since most of the ^{14}C-labeled material produced in incubations with high concentrations of actinomycin D migrated close to the solvent front and was not actinomycin D. In a similar experiment, using radiolabeled L-valine as substrate, the presence of actinomycin D caused a similar inhibition of activity.

Actinomycin synthesis by mycelium was not similarly affected by addition of actinomycin D to the incubation mixtures (Table 4). In fact, under conditions where mycelium was preincubated for 2 h in the protoplast buffer (procedure B), the incorporation of radiolabel into actinomycin increased somewhat with increasing amounts of actinomycin D present in the assay.

(iii) Effect of amino acid analogs. It was expected that the protoplast system might be more amenable to the study of intermediates in actinomycin synthesis than was intact mycelium, since intracellular compounds can be easily released from protoplasts by osmotic lysis. D-Valine was previously shown to be a strong inhibitor of actinomycin synthesis with intact cells of *S. antibioticus* (19); moreover, this inhibition by D-valine resulted in the accumulation of a metabolite, 4-methyl-3-hydroxyanthranilic acid (4), that appears to be a direct precursor of the actinomycin chromophore, actinocin (15, 35). Therefore, it was of interest to ascertain whether D-valine inhibited actinomycin synthesis and brought about the accumulation of 4-methyl-3-hydroxyanthranilic acid or other metabolites by protoplasts.

As noted previously with *S. antibioticus*, D-valine was found to inhibit synthesis of actinomycin by *S. parvulus* mycelium (Table 5). The results shown were obtained with [*methyl*-^{14}C]methionine as precursor, but we noted similar findings with L-[^{14}C]proline or L-[^{14}C]valine. However, when protoplasts were incubated with various concentrations of D-valine with one of these precursors, there was surprisingly little inhibition. It seemed unlikely that intermediates would accumulate under these conditions.

Two other analogs (α-methyl-DL-tryptophan

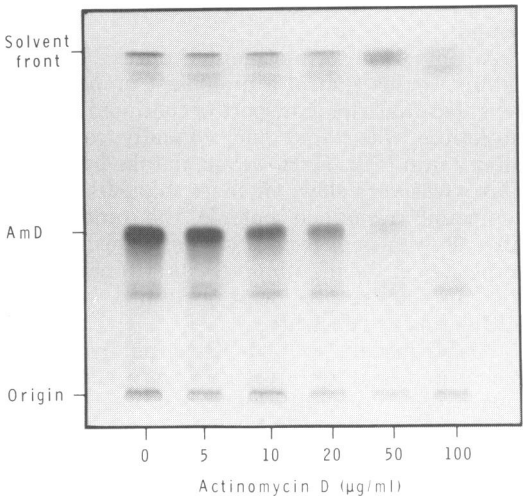

FIG. 6. *Effect of actinomycin D (AmD) on synthesis of actinomycin by protoplasts. Protoplasts were incubated with AmD for 10 min before addition of [methyl-^{14}C]methionine. After 1 h, incubations were extracted with ethyl acetate, and the extractable material was analyzed by TLC and radioautography as noted in the text.*

TABLE 5. *Effect of D-valine on actinomycin synthesis by mycelium and protoplasts*[a]

D-valine concn (M)	Protoplasts		Mycelium	
	cpm extracted/ incubation	% Control	cpm extracted/ incubation	% Control
0	11,310	100	12,240	100
10^{-4}	10,420	92	2,045	17
10^{-3}	9,510	84	1,480	12
10^{-2}	9,630	85	1,100	9

[a] Mycelium (procedure A) or protoplasts were incubated with D-valine at the concentration shown for 10 min before addition of L-[*methyl*-^{14}C]methionine.

and *cis*-3-methyl-DL-proline) that are known to inhibit actinomycin synthesis by intact *S. antibioticus* mycelium (22, 37, 41) were also tested with mycelium and protoplasts of *S. parvulus*. As with D-valine, it was found that actinomycin synthesis by mycelium was much more sensitive than that of protoplasts to inhibition by both of these analogs.

(iv) Uptake of amino acids. As stated earlier, the kinetics of [*methyl*-^{14}C]methionine incorporation into actinomycin by protoplasts suggested that they had a decreased ability to take up the amino acid. Also, the significantly larger concentrations of an amino acid analog needed for inhibition of actinomycin synthesis by protoplasts, in contrast with those needed for inhibition in mycelium, might also be attributed to a decreased uptake of these compounds.

A comparison of methionine uptake by mycelium versus that of a protoplast preparation revealed that the transport of methionine into mycelium was rapid and virtually complete after 2 min (Fig. 7). However, uptake by protoplasts was very slow, for more than 80% of the compound remained outside the protoplasts after 15 min and, even after a 2-h incubation, some 30% of the methionine was still not taken up. Similar results were obtained with L-[^{14}C]valine as substrate.

The transport of D-[1-^{14}C]valine by protoplasts and that by mycelium were also compared. With mycelium, uptake of D-valine (0.1 mM) was initially rapid (about 16 nmol/mg of protein per min) and then continued at a decreased rate. By comparison, no uptake of a similar amount of D-valine by protoplasts was observed, even after a 2-h incubation.

A similar study was also carried out with sarcosine. As noted previously (7), uptake of [^{14}C]sarcosine (0.01 mM) by mycelium was relatively slow when compared with the entry of methionine, for about 10% was removed from the medium after 15 min of incubation, and only about 45% was taken up after 3 h. Analysis by TLC and autoradiography of material in the ethyl acetate extract from this incubation showed the presence of two radiolabeled compounds, which comigrated with actinomycins II and III. By contrast, uptake of radiolabeled sarcosine (1.3 mM) by protoplasts was not detected, even after a 2-h incubation, and this correlated with the finding that no ^{14}C-labeled actinomycin was produced under these conditions (see above).

The effect of exogenously supplied actinomycin D on actinomycin synthesis by protoplasts (noted above) appears to be due, in part, to an inhibition of transport of the ^{14}C-amino acid precursor. Examination of the uptake of methionine revealed that disappearance of [*methyl*-^{14}C]methionine was reduced by 30 to 35% in the presence of actinomycin D (50 μg/ml); similarly, uptake of L-[^{14}C]valine was reduced by 50 to 55% under these conditions.

As determined microscopically, protoplasts incubated with actinomycin D (50 μg/ml) for 1 or 2 h did not appear to undergo lysis and, in fact, were indistinguishable from protoplasts incubated without added actinomycin.

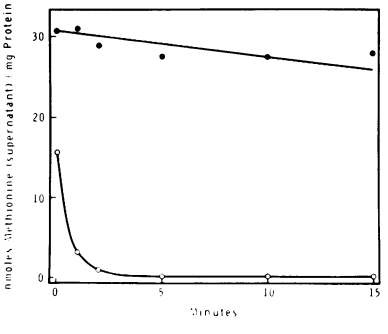

FIG. 7. *Uptake of [methyl-^{14}C]methionine by protoplasts and mycelium. Incubations were carried out as described in the text, and the amount of [methyl-^{14}C]methionine remaining in the filtrate was determined. For the experiment with mycelium (○) the concentrations of protein and methionine were 0.56 mg/ml and 10 μM, respectively (17.9 nmol of methionine per mg of protein). For the experiment with protoplasts (●) the concentrations of protein and methionine were 1.62 mg/ml and 51 μM, respectively (31.5 nmol of methionine per mg of protein).*

DISCUSSION

Protoplasts from *S. parvulus* can be produced by treatment of mycelium with lysozyme in a stabilizing buffer solution similar to that used to prepare protoplasts from *S. griseus* and *S. venezuelae* (32). Moreover, protoplasts obtained from actinomycin-producing mycelium of *S. parvulus* are also capable of de novo antibiotic synthesis. This is evident from the facts that the radiolabel from all the precursor amino acids was incorporated into actinomycin D and that the ^{14}C radiolabel from each precursor was located in the molecule at the sites expected. There appeared to be significant specificity with regard to the radiolabeled amino acids (L-tryptophan, benzene ring moiety; L-methionine; glycine; L-proline; L-threonine; and L-valine) that served as precursors of actinomycin synthesis. L-Lysine and L-aspartic acid, nonprecursor amino acids, were not utilized for this purpose. Incorporation of radiolabel from D-valine into ethyl acetate-extractable material was so slight that it was not possible to estab-

lish whether the radioactivity was specifically incorporated into actinomycin D. However, earlier experiments with intact mycelium demonstrated that L-valine (but not the D-enantiomer) is the precursor of the D-valine in actinomycin (23, 29). Oxidative deamination of the D-amino acid followed by transamination of the corresponding keto acid to L-valine might provide small amounts of the appropriate precursor of the D-valine and *N*-methyl-L-valine components in the antibiotic.

Several observations enable us to conclude that the activity measured is due to the synthesis of actinomycin by protoplasts rather than by small mycelial fragments that, possibly, may have passed through the glass wool filter during preparation of the protoplast suspensions. First, the synthesizing activity of protoplasts is sensitive to a decrease in sucrose concentration; on the other hand, the activity of mycelium is not. Second, the activity of the protoplast system is much less susceptible to inhibition by D-valine, *cis*-3-methylproline, and α-methyl-DL-tryptophan. This may be due to reduced uptake of these compounds, as the rate of transport of several amino acids (methionine, sarcosine, and L- and D-valine) into protoplasts was observed to be much lower than that into mycelium. It may be that a number of peripheral proteins concerned with transport are lost from the plasma membrane when the cell wall is removed through the action of lysozyme. In other organisms, removal of the cell wall with lysozyme has been shown to cause loss of enzymes and proteins associated with transport (2, 14, 16). Third, the effects of addition of actinomycin D on synthesis of actinomycin by protoplasts and by mycelium differ. With respect to mycelium, exogenous actinomycin D had no adverse affect on de novo antibiotic synthesis. By contrast, synthesis of actinomycin by protoplasts is severely diminished by the presence of actinomycin D; at 50 μg/ml, formation of the antibiotic was inhibited at least 80%. Inhibition of transport of the labeled precursor does not appear to be the sole reason for the reduction in synthesis, as uptake by protoplasts of methionine and valine was reduced only about 35 and 55%, respectively. Conceivably, actinomycin can inhibit its own synthesis in protoplasts by a mechanism analogous to feedback inhibition. Previous studies (6, 9, 42; Hughes et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, O47, p. 253) have led to the hypothesis that antibiotic-producing organisms develop an 'immunity system' during the idiophase, when the antibiotic is being formed. The observed difference in response between protoplasts and mycelium to actinomycin D suggests that the immunity system may be lost or modified during protoplast formation and, therefore, may have a peripheral location, either in or on the wall or plasma membrane or between them.

The protoplast system may be of limited value in the study of in vivo antibiotic synthesis, as the use of mycelium per se requires less preparation, comprises an osmotically stable system, is more active, and is much more responsive to the effects of substrates and inhibitors. As noted above, uptake of substrates (as well as inhibitors) by protoplasts is lower than that by the mycelium. The failure by protoplasts to utilize sarcosine for actinomycin synthesis appears to be due to the absence of a functional transport system for the amino acid.

Despite these limitations, protoplasts may prove useful in a number of other investigations. For example, it may be possible to study the accumulation of intermediates of antibiotic synthesis. The unknown metabolite derived from L-tryptophan in the incubations with protoplasts has not been found with mycelium and may potentially be an intermediate in actinomycin biosynthesis. Although the use of inhibitors was relatively ineffective in the present experiments with protoplasts, their effect upon antibiotic synthesis might be examined by preloading mycelium with an inhibitor before protoplast formation. Conceivably, intermediates of actinomycin synthesis might be detected by this means, as previously found with *S. antibioticus* mycelium (4). As demonstrated with *B. licheniformis* (18), osmotic disruption of protoplasts could provide an effective route for obtaining an in vitro actinomycin-synthesizing system. Furthermore, protoplasts may also be useful in studies of the development of resistance by an antibiotic-producing species to its own antibiotic, and they appear to be suitable for investigations of genetic exchange via protoplast fusion (10, 17, 36) with antibiotic-producing microorganisms.

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