Development of a Chemically Defined Medium for the Synthesis of Actinomycin D by *Streptomyces parvulus*

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A chemically defined medium, consisting of D-fructose, L-glutamic acid, L-histidine, K_2HPO_4, $MgSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, $FeSO_4 \cdot 7H_2O$, $CoCl_2 \cdot 6H_2O$, and deionized water, was developed for synthesis of high yields (500 to 600 $\mu g/ml$) of actinomycin D by *Streptomyces parvulus*. Under these nutritional conditions, growth and actinomycin formation did not follow a typical trophophase-idiophase pattern. The amino acids appeared to have a sparing action on the utilization of D-fructose which was slowly and incompletely metabolized during mycelium development and antibiotic production. A significant repression of actinomycin synthesis by *S. parvulus* was observed when D-glucose (0.01 to 0.25%) was added to the culture medium. The repression was not due to a decline in the pH of the medium during glucose catabolism.

Because of our continued interest in the mechanism of directed biosynthesis (30), we have turned our attention to the metabolic activities of *Streptomyces parvulus* which synthesizes virtually the single antibiotic component, actinomycin D (40). Although the medium described previously (16, 32) proved satisfactory for the production of actinomycin mixtures by *Streptomyces antibioticus*, the synthesis of actinomycin D by *S. parvulus*, under the same conditions, was rather poor and extremely variable. Studies were, therefore, initiated to establish the nutritional factors that would support growth and consistently high yields of the antibiotic by this organism. This report describes a series of experiments that have culminated in the development of a chemically defined medium in which production of high titers of actinomycin D was achieved.

MATERIALS AND METHODS

Organism and conditions of cultivation. *S. parvulus* (ATCC 12434) was maintained on slants of glucose-yeast extract-malt extract agar medium (26). Incubation was carried out for 3 to 5 days at 30°C until gray spores had developed; slants were then kept at 4°C for further use.

For preparation of vegetative inoculum, spores were scraped gently from the surface of a slant(s) with 2.5 ml of NZ-Amine medium (31), and 2-ml quantities of the spore suspension were inoculated into a 250-ml Erlenmeyer flask containing 100-ml amounts of NZ-Amine medium. After incubation on a gyratory shaking incubator (New Brunswick Scientific Co., Inc., New Brunswick, N.J.) for 48 h at

30°C, the mycelium was harvested by centrifugation (30 min at 9,000 rev/min), washed twice with and then suspended in 100 ml of saline as described previously (31, 34). Aliquots (3 ml) of the suspension served as inoculum for each 250-ml Erlenmeyer flask containing 100 ml of chemically defined medium.

Basal medium for nutritional studies. The medium (D-galactose, 10 g; D-glucose, 1.0 g; L-glutamic acid · HCl, 2.5 g; $MgSO_4 \cdot 7H_2O$, 25 mg; $ZnSO_4 \cdot 7H_2O$, 25 mg; $CaCl_2 \cdot 2H_2O$, 25 mg; $FeSO_4 \cdot 7H_2O$, 25 mg; deionized water, 1,000 ml; pH 7.1) routinely utilized for actinomycin production with *S. antibioticus* and *Streptomyces chrysomallus* (16, 33, 34, 60) was employed initially for the studies with *S. parvulus*.

Carbon sources (e.g., sugars, organic acids) generally were sterilized separately and added at appropriate concentrations just prior to inoculation. All other additions were made before sterilization of media. Incubations were carried out in duplicate on a gyratory shaking incubator at 250 rev/min for 6 days at 30°C.

Analytical methods. Growth of the culture was followed by measurement of mycelial dry weight (35). The actinomycin titer per milliliter of culture filtrate was determined spectrophotometrically at 443 nm (34) on samples taken after 2, 4, and 6 days of incubation. The data reported in most experiments represent the yield of actinomycin (in micrograms per milliliter) after 6 days of cultivation.

Residual D-fructose was measured enzymatically as outlined by Bernt and Bergmeyer (5). The culture filtrate (5 ml) was shaken for 20 min with Amberlite MB-3 resin (200 mg, 20 to 50 mesh) in a ground glass-stoppered test tube and then centrifuged for 10 min at 2,000 rev/min. Determination of fructose concentration was then performed in triplicate on aliquots of the supernatant. Examination of the fruc-

tose preparation for glucose was performed with the Glucostat reagent (16); these analyses revealed that fructose was essentially free of glucose (<0.01%).

Disappearance of L-glutamic acid and L-histidine from the medium was followed by the paper chromatographic procedure of Naftalin (41) and by means of the automatic analytical Beckman Spinco amino acid analyzer (model 120C) (58, 59). An aliquot of the culture filtrate (5 ml, adjusted to pH 2.5 to 3.0) was passed through a Dowex 50W column (1 by 6 cm, H$^+$ form, 200 to 400 mesh), and the amino acids were eluted with 2 N NH$_4$OH (34). The solution was evaporated to dryness and then employed for the Naftalin and amino acid analyzer procedures.

Chromatography and high-voltage electrophoresis. Aliquots of each sample in water were examined by the Naftalin method (41), employing ascending paper chromatography (Whatman no. 1) and the solvent system: 1-butanol:acetic acid:water (12:3:5); the R_f values for L-glutamic acid and L-histidine were 0.29 and 0.11, respectively. For the analytical determinations with the Beckman analyzer, samples were diluted in 0.2 N sodium citrate buffer, pH 2.2, and developed in 0.2 M sodium citrate buffer, pH 3.05, for L-glutamic acid, or 0.35 M sodium citrate, pH 5.25, for L-histidine (basic column). The flow rate of buffer for L-glutamic acid was 68 ml/h and for L-histidine it was 34 ml/h; the flow rate for ninhydrin throughout was 34 ml/h. Retention time for L-glutamic acid was 90 min and for L-histidine it was 43 min.

D-Fructose ($R_f = 0.49$) was examined by descending paper chromatography overnight with Whatman no. 1 paper, employing the solvent system ethyl acetate:pyridine:water (3.6:1:1.15). Detection was effected with a silver nitrate reagent (52); the chromatographic study revealed that the fructose preparation was homogeneous.

The actinomycin produced by S. parvulus was routinely monitored by means of circular paper chromatography as described in earlier publications (31–33). The solvent systems employed were (i) 10% aqueous sodium-o-cresotinate:dibutyl ether:sym, tetrachloroethane (4:3:1); (ii) 10% aqueous sodium-o-cresotinate: isopropyl ether:chloroform (6:5:1) and (5:3:2); and (iii) 5% aqueous sodium-o-cresotinate:amyl acetate, unequilibrated.

Amino acid hydrolysates (34) of actinomycin preparations were examined by a two-dimensional technique (59) employing high-voltage electrophoresis in one direction and paper chromatography (methanol, water, pyridine [20:5:1]) in the second direction. Amino acids were visualized with 0.2% ninhydrin/acetone; imino acids also were detected with 0.2% isatin/acetone.

L-Histidine, D-fructose, and hexokinase (EC 2.7.1.1) were purchased from Calbiochem (San Diego, Calif.). Adenosine-5'-triphosphate, nicotinamide adenine dinucleotide phosphate, glucose-6-phosphate dehydrogenase (EC 1.1.1.49), and phosphoglucose isomerase (EC 5.3.1.9) were obtained from Sigma Chemical Co. (St. Louis, Mo.). L-Glutamic acid·HCl was purchased from Mann Research Lab. (New York, N.Y.); D-glucose was from Fisher

Scientific Co. (Pittsburgh, Pa.); and the Glucostat reagent kit was from Worthington Biochemical Corp. (Freehold, N.J.). The Dowex 50W resin (H$^+$ form, 200 to 400 mesh) was procured from BioRad Lab (Richmond, Calif.) and the Amberlite MB-3 monobed resin was obtained from Mallinckrodt Chemical Works (St. Louis, Mo.). All other compounds used were purchased from commercial sources.

RESULTS

Nature of actinomycin produced by S. parvulus. The qualitative nature of the actinomycin (predominantly actinomycin D, >95%) elaborated by S. parvulus during the period of medium development did not change significantly from that normally elaborated by the organism as monitored by circular paper chromatography, spectrophotometry, and analyses of the amino acid composition of acid hydrolysates of actinomycin preparations.

Sources of carbohydrates. A number of carbohydrates were investigated as carbon sources for actinomycin production in the glutamic acid-basal mineral salts medium. As control, the same medium was employed with galactose (1%) and glucose (0.1%) as carbohydrate sources. Preliminary experiments revealed that, of a large number of compounds tested, D-fructose (191 μg/ml), D-galactose (128 μg/ml), dextrin (127 μg/ml), and D-glucose (104 μg/ml) singly supported increased yields of actinomycin in comparison with the control medium (87 μg/ml). These four carbohydrates were then retested at a concentration of 0.5 and 1.0%. It can be seen (Fig. 1) that the level of the carbon compound employed had a striking influence on actinomycin production; D-fructose was far superior to the other carbohydrates tested at either concentration. In contrast to our preliminary experiments, actinomycin titers were extremely low when D-glucose served as the sole carbohydrate source.

Effect of different amino acids. With D-fructose (1%) present, various amino acids (5 mM) were examined in lieu of L-glutamic acid for antibiotic production. Several amino acids (e.g., valine, histidine, ornithine, glutamine, asparagine, and leucine) appeared suitable for the biosynthesis of actinomycin D (Table 1). L-Glutamic acid, at 5 mM, was rather ineffective as a nitrogen source; however, at a level of 15 mM, it proved to be an excellent nitrogen source for antibiotic formation (160 μg/ml).

These results prompted an experiment to determine the optimal concentration of selected amino acids (L-valine, L-ornithine, L-glutamic acid, L-histidine, L-proline, and L-glutamine) as sole nitrogen source for actinomycin synthesis. The amino acids were used within the concen-

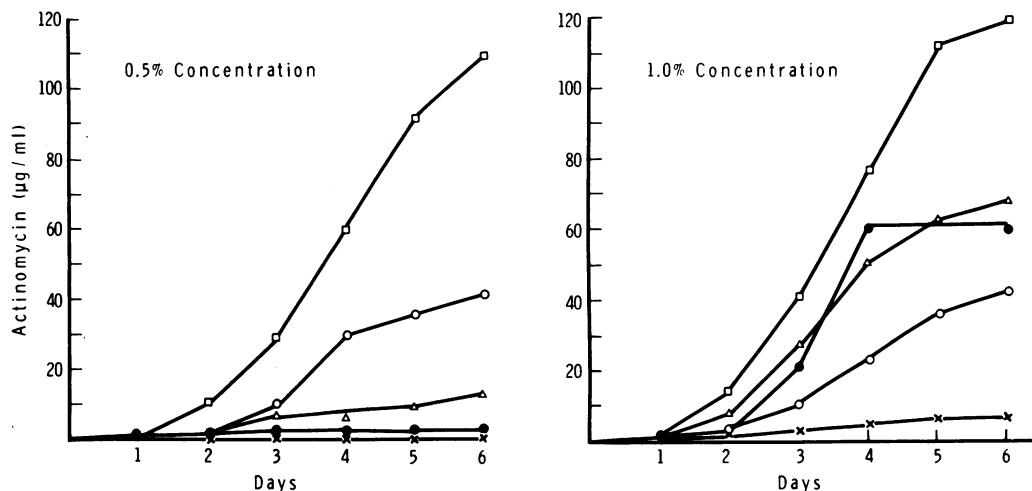CARBOHYDRATES AS CARBON SOURCES

FIG. 1. *Influence of various carbohydrates upon actinomycin synthesis by Streptomyces parvulus in L-glutamic acid-basal mineral salts medium (16). Carbohydrates were employed at the 0.5 and 1 % level, except for the control medium. Symbols:* □, D-Fructose; △, D-galactose; ●, dextrin; ×, D-glucose; ○, control (D-galactose, 1.0%; and D-glucose, 0.1%).

tration range of 3 to 30 mM with D-fructose (1%) and the basal mineral salts; the results are presented in Fig. 2 as a histogram. As seen, histidine (5 mM), ornithine (10 mM), valine (10 mM), glutamic acid (15 mM), glutamine (10 mM), and proline (15 mM) provided the optimum level of each amino acid for actinomycin production. Of interest is the fact that higher concentrations of these amino acids were less effective for antibiotic synthesis. We then compared the effect of L-valine, L-proline, L-ornithine, and L-histidine on actinomycin production when used in combination with L-glutamic acid (15 mM) in the chemically defined medium. The data (Table 2) reveal that antibiotic formation was enhanced if the amino acid in question was combined with glutamic acid as nitrogen source. In particular, valine (3 to 5 mM) and histidine (3 to 5 mM) were the most suitable ancillary amino acids for synthesis of actinomycin.

Influence of fructose concentration upon actinomycin formation. The influence of D-fructose concentration on actinomycin formation was next explored in a medium containing the basal mineral salts, glutamic acid (15 mM) and varying levels of valine or histidine. As shown in Fig. 3, (i) actinomycin synthesis increased as a function of the fructose concentration supplied in the medium, and (ii) the combination of histidine and glutamic acid was somewhat superior to a similar mixture of valine and glutamate for antibiotic production.

Effect of inorganic nitrogen sources. Inorganic nitrates, nitrite, and several ammonium salts were also investigated as sole nitrogen sources for the formation of actinomycin D. The concentrations employed ranged from approximately one-fourth to twice the amount of nitrogen contained in the glutamic acid-histidine medium. At the levels employed, however, none of these compounds were as effective as the combination of glutamic acid and histidine; the maximal titers of actinomycin D attained were one-third that produced with the amino acids present.

Role of organic acids in the production of actinomycin. Although some investigations have shown that organic acids are poor sources of carbon for production of antibiotics when used singly (12, 13), other studies have revealed that the combination of a sugar and the sodium salt of an organic acid produced antibiotic titers higher than those elaborated with these carbon sources used singly (1, 18, 24, 27, 28). Since D-fructose proved to be an excellent carbohydrate for antibiotic production, we examined the role of organic acids (0.1 to 0.5%) as supplemental carbon sources for actinomycin synthesis. Generally, most of the organic acids tested at levels above 0.1% were inhibitory to actinomycin formation.

Effect of carbon-nitrogen ratio on actinomycin formation. To further explore the influence of the organic nutrients upon actinomycin synthesis, the concentration of D-fructose (4%)

TABLE 1. *Effect of various amino acids on actinomycin production by Streptomyces parvulus* [a]

Amino acid	Actinomycin (μg/ml)
L-Glutamic acid · HCl (control) [b]	160
L-Valine	153
L-Histidine	132
L-Ornithine · HCl	124
L-Glutamine	109
L-Asparagine · HCl	105
L-Leucine	104
L-Proline	95
L-Serine	91
L-Alanine	90
β-Alanine	89
L-Threonine	87
L-Aspartic acid	79
L-Citrulline	79
L-Phenylalanine	69
L-Glutamic acid · HCl	65
L-Tyrosine	63
L-Isoleucine	58
L-Lysine · HCl	49
Glycine	47
L-Methionine	37
L-Tryptophan	28
L-Cysteine · HCl	28
L-Cystine	23
L-Arginine · HCl	23

[a] *S. parvulus* was grown in a 1% D-fructose-basal mineral salts medium for 144 h at 30°C.

[b] L-Glutamic acid · HCl was supplied at 15 mM (control); all other amino acids were employed at 5 mM.

was held constant, while the amount of L-glutamic acid and L-histidine was varied to provide different C/N ratios in the medium. The results of these experiments are depicted in Fig. 4. Maximum formation of actinomycin (455 μg/ml) was achieved at a C/N ratio of 41.7. This ratio corresponded to 4% D-fructose, 15 mM L-glutamic acid, and 5 mM L-histidine.

Mineral requirements. Several laboratories have noted that various cations, such as Mg^{2+}, Fe^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+}, and Co^{2+} are stimulatory to antibiotic production (55). In some instances rigorous methods of purification (e.g., chelation or adsorption) have been employed to free medium constituents from trace metal contamination (1, 4, 8, 39). In other cases no special precautions have been made; the effects of exogenously provided trace elements have been examined over and above that present as contaminants on glassware, chemicals, etc. (9, 18, 47).

Role of trace metals. The relative requirement of several individual microelements for actinomycin production was studied in the medium consisting of: D-fructose, 40.0 g; L-glutamic acid, 15 mM; L-histidine, 5 mM; K_2HPO_4,

1.0 g; $MgSO_4 \cdot 7H_2O$, 25 mg; $ZnSO_4 \cdot 7H_2O$, 25 mg; $CaCl_2 \cdot 2H_2O$, 25 mg; $FeSO_4 \cdot 7H_2O$, 25 mg; and deionized water, 1,000 ml with a pH 7.1. The trace metal under test was added to the basal medium at different concentrations in separate flasks. In the subsequent experiments, the composition of the medium was modified to include this amount of the trace element. Figures 5 to 8 reveal that the optimal concentrations of the various cations tested were 4.88 ppm of Mg^{2+}, 8 to 10 ppm of Fe^{2+}, 7.5 to 14.6 ppm of Ca^{2+}, and 4.6 to 13.7 ppm of Zn^{2+}. The optimal range appeared somewhat broader for Ca^{2+} and Zn^{2+}.

Fosfomycin biosynthesis by *Streptomyces fradiae* (47), gentamicin formation by *Micromonospora* species (R. T. Testa, personal communication), as well as the production of certain other antibiotics by streptomycetes have been found to be stimulated or influenced by the addition of Co^{2+} to the production medium (9, 45). An experiment was carried out to determine whether Co^{2+} would exert a similar effect on actinomycin synthesis by *S. parvulus*. The results of two experiments suggest that Co^{2+} may stimulate antibiotic production (10 to 25%), particularly at a concentration of $CoCl_2 \cdot 6H_2O$ of 1×10^{-7} M to 5×10^{-8} M.

Influence of K_2HPO_4 concentration. The concentration of K_2HPO_4 found optimal for actinomycin production in the chemically defined medium was 0.1% (565 μg/ml), although the level of the inorganic compound that could be employed for excellent synthesis ranged from 0.05 to 0.4%. Even at a concentration of 1.0%, production was still three-fourths that observed at the optimal concentration of K_2HPO_4. Acti-
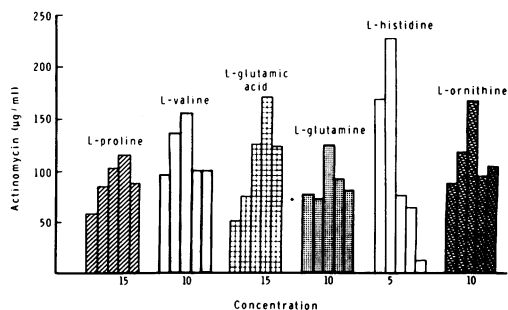

FIG. 2. *Histogram depicting the influence of the concentration of different amino acids upon actinomycin formation in a medium containing 1% D-fructose and basal mineral salts. Concentration of each amino acid (L-proline, L-valine, L-glutamic acid, L-glutamine, L-histidine, and L-ornithine), reading from left to right, was 3, 5, 10, 15, and 30 mM. Incubation was at 30°C for 144 h. The numerical values shown represent the optimal concentration of each amino acid for actinomycin production.*

TABLE 2. *Effect of L-amino acids provided singly or in combination with L-glutamic acid for actinomycin synthesis by S. parvulus* [a]

L-Glutamic acid (mM)	L-Valine (mM)	L-Ornithine (mM)	L-Histidine (mM)	L-Proline (mM)	Actinomycin (μg/ml)	% of control
15	0	0	0	0	170	1.00
0	3	0	0	0	75	0.44
15	3	0	0	0	190	1.12
0	5	0	0	0	115	0.68
15	5	0	0	0	215	1.26
0	10	0	0	0	126	0.74
15	10	0	0	0	165	0.97
0	0	3	0	0	75	0.44
15	0	3	0	0	160	0.94
0	0	5	0	0	105	0.62
15	0	5	0	0	177	1.04
0	0	10	0	0	85	0.50
15	0	10	0	0	165	0.97
0	0	0	3	0	155	0.91
15	0	0	3	0	215	1.26
0	0	0	5	0	140	0.82
15	0	0	5	0	210	1.24
0	0	0	10	0	55	0.32
15	0	0	10	0	195	1.15
0	0	0	0	3	90	0.53
15	0	0	0	3	155	0.91
0	0	0	0	5	140	0.82
15	0	0	0	5	140	0.82
0	0	0	0	10	110	0.65
15	0	0	0	10	140	0.82

[a] Conditions were the same as in Table 1, except incubation was for 135 h at 30°C. Media contained D-fructose (1%).

nomycin synthesis appears to be far less sensitive to the inorganic phosphate concentration in the medium than the corresponding fermentations of prodigiosin, monamycin, pyocyanine, or vancomycin (57). By contrast, Haavik (21) reported that optimal synthesis of bacitracin was observed at levels of inorganic phosphate in excess of 100 mM, which represents a 20-fold greater amount than required for maximal actinomycin formation.

Repression of actinomycin synthesis by D-glucose. The repressive effect of glucose and related compounds on enzyme synthesis and secondary metabolite formation has been discussed in several reviews (10, 36, 42) and the mechanism of catabolite repression has been critically examined (46). In the case of *S. antibioticus*, glucose markedly repressed synthesis of actinomycin as well as phenoxazinone synthase, a key enzyme required for the formation of the actinomycin chromophore, actinocin (16). It was postulated that the repression of antibiotic synthesis observed with glucose may be due primarily to a repression of the formation of

phenoxazinone synthase and other key enzymes required for antibiotic biosynthesis.

Glucose was a poor carbon source when employed as the sole carbohydrate for synthesis of actinomycin (Fig. 1). It significantly repressed antibiotic formation in the fructose-glutamic acid-histidine-mineral salts medium (Fig. 9). Acidity was not responsible for the reduced synthesis of antibiotic since the pH of the media containing glucose (0.01 to 0.25%) was similar to that of the control culture. These data also contrast with the findings of Haavik (20, 22). Formation of bacitracin was not considered to be under catabolite repression control by glucose; the inhibitory nature of the sugar was believed to be due to the low pH created during the first few hours of incubation.

Biochemical analyses of substrate utilization during growth and antibiotic production. A study of the dynamics of the fermentation process was carried out to determine the extent to which the organic substrates were consumed during growth and production of actinomycin by the organism. As shown in Fig. 10, disap-
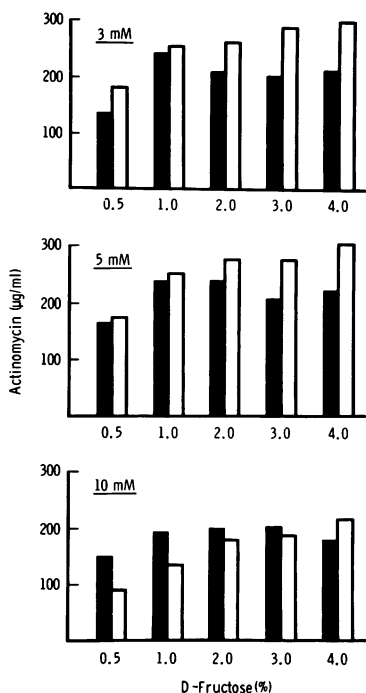
FIG. 3. *Effect of D-fructose concentration upon actinomycin formation in a medium containing basal mineral salts and L-glutamic acid (15 mM) in combination with L-valine or L-histidine employed at 3, 5, or 10 mM. Incubation was for 144 h at 30°C. Solid bars represent L-valine and open bars represent L-histidine.*

pearance of the amino acid substrates was extremely rapid with 50% of histidine and glutamic acid taken up by the cells within 12 h; the amino acids were removed (>99%) by 18 to 20 h, i.e., shortly before the onset of actinomycin production. By contrast, the utilization of fructose was a much slower process. Enzyme assays (5) indicated that there was an initial lag of approximately 8 h before it was taken up by the organism; however, even after 140 h, only 30% of the sugar supplied initially had been utilized.

Growth of the culture and production of the antibiotic did not appear to follow the typical pattern of a trophophase-idiophase fermentation (7, 10, 56). After an initial 3-h lag, there was a rapid increase of mycelium until 48 h; during the ensuing incubation, however, the organism continued to grow slowly until the experiment was terminated. Actinomycin was detected in the culture medium after 24 h; subsequently, its formation and growth of the culture remained parallel for the duration of the experiment.

DISCUSSION

The major purpose of this investigation was to design a chemically defined medium suitable for the growth and for the production of high titers of actinomycin by *S. parvulus*. The data described herein do reveal that yields of 500 to 600 μg of actinomycin D per ml can be consistently obtained by the organism in a glutamic acid-histidine-fructose-mineral salts medium.

Examination of a variety of carbohydrate sources for antibiotic production has revealed that D-fructose is far more effective than any of the other substances examined. Experimentally, we observed that a concentration of 4% was optimal for actinomycin production; however, the analytical results (Fig. 10) reveal that the utilization of the hexose occurred slowly and that only 30% (1.2 g) was consumed by 140 h, i.e., at the termination of the experiment. Similar conclusions were reported by Parit-
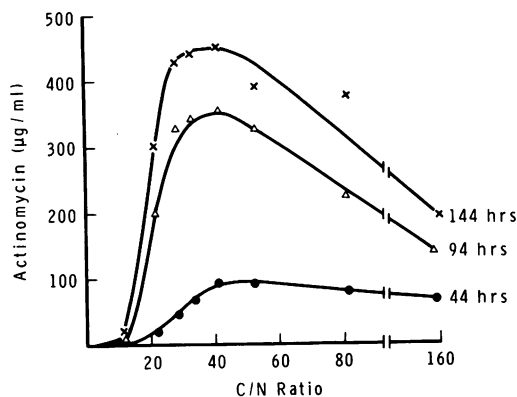

FIG. 4. *Influence of carbon-nitrogen ratio on actinomycin synthesis. S. parvulus was grown in media containing basal mineral salts with 4% D-fructose and varying amounts of L-glutamic acid and L-histidine as shown below:*

L-Glutamic acid (mM)	L-Histidine (mM)	D-Fructose (%)	C/N
3.75	1.25	4	155.3
7.5	2.5	4	79.2
11.25	3.75	4	53.8
15.0	5.0	4	41.7
18.6	6.25	4	33.5
22.5	7.5	4	28.4
30.0	10.0	4	22.0
60.0	20.0	4	12.5

The carbon-nitrogen ratio is based on the following calculations:

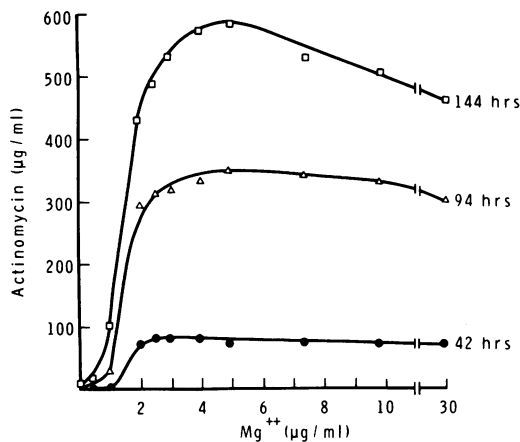| Determination	C(%)	N(%)
D-Fructose	40	
L-Glutamic acid	40.8	9.5
L-Histidine	46.4	27.1

FIG. 5. Influence of Mg^{2+} ion upon actinomycin production after 42, 94, and 144 h of incubation.
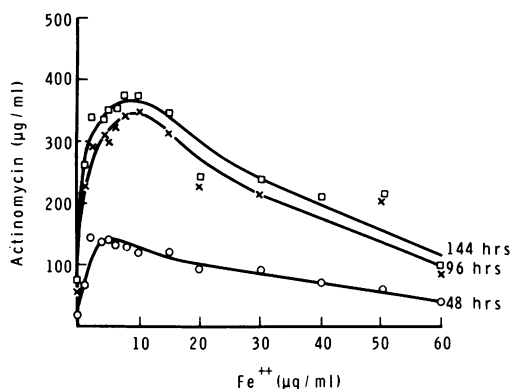

FIG. 6. Influence of Fe^{2+} ion upon actinomycin production after 48, 96, and 144 h of incubation.

The uptake of these two amino acids by S. parvulus was found to occur quite rapidly, with 50% of the histidine and glutamic acid taken up within 12 h and essentially both amino acids (>99%) being removed by 18 to 20 h. The data in Fig. 10 suggest that the amino acids are preferentially employed initially for cell growth and that they exert a sparing effect on the utilization of the hexose. Moreover, the enzymes involved in the transport (44, 54) as well as the subsequent metabolism of fructose (e.g., fructokinase) (49, 50) may be inducible and require time for synthesis before the sugar can be metabolized.

The metabolic fate of glutamic acid and histidine, once they are taken up intracellularly, has not been investigated as yet. L-Glutamic acid has been shown to be a highly favorable source of nitrogen and carbon for growth of Streptomyces (12, 17, 48) and for the production of several chemically different antibiotics (24, 38, 39, 47). It also serves as a precursor of L-proline (2), which is an important constituent of
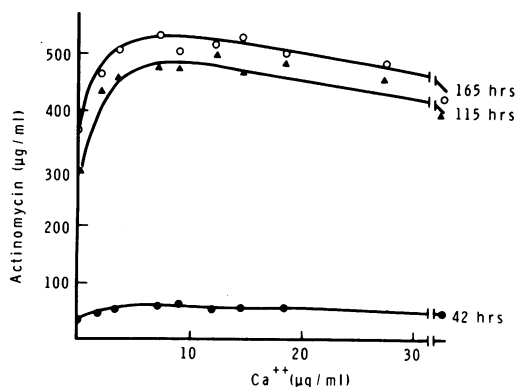
skaya et al. (43) who examined the growth of Actinomyces roseoflavus in a fructose-containing medium. They noted that not more than 10 to 15% of the fructose present in the medium was oxidized irrespective of the initial concentration supplied; addition of mannitol to the fructose-containing medium improved the utilization of fructose to some extent (22 to 27%) (14). The slow utilization of D-fructose by S. parvulus throughout the fermentation ensures that a carbon and energy source was available during the period of antibiotic production. It should be pointed out that fructose is also an excellent source of carbon for gramicidin S formation (53).

Although a number of L-amino acids were favorable sources of nitrogen (and carbon) for actinomycin synthesis, when used singly, the combination of L-histidine (5 mM) and L-glutamic acid (15 mM) in the D-fructose-containing medium yielded the highest actinomycin titers.

FIG. 7. Effect of Ca^{2+} ion upon actinomycin synthesis after 42, 115, and 165 h of incubation.
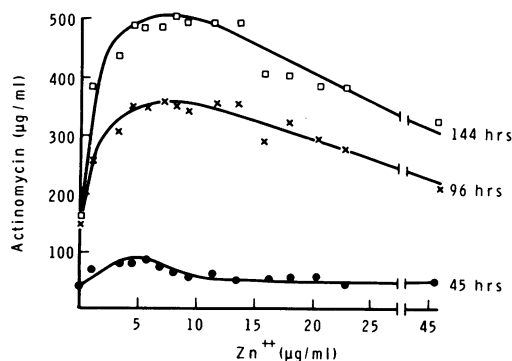

FIG. 8. Effect of Zn^{2+} ion upon actinomycin formation after 45, 96, and 144 h of incubation.

the actinomycin pentapeptides (40). L-Histidine has been reported to serve as a source of nitrogen for candicidin production (1). Moreover, it has proved useful in combination with valine and arginine for the formation of streptomycin; Eiser and McFarlane (15) observed that there was a direct correlation between the histidine

concentration in the medium and streptomycin production. With regard to *S. parvulus,* the effectiveness of L-histidine may be due in part to its ability to serve as (i) a buffer, (ii) a chelating agent, (iii) a direct source of the amino acid for protein synthesis, and (iv) an indirect source of L-proline through its catabolism to glutamic acid (19, 37).

As the data in Fig. 10 further reveal, growth of *S. parvulus* and production of the antibiotic do not follow the typical pattern of a trophophase-idiophase fermentation (7, 10, 55, 56). After a short lag (3 h), growth of the organism increased rapidly, initially at the expense of the amino acid substrates in the medium. Antibiotic synthesis was observed after 24 h of incubation; however, further growth of the organism and production of the antibiotic continued more or less in parallel throughout the remainder of the incubation. Presumably, D-fructose was then utilized as the chief carbon and energy source, and the intracellular pool provided the nitrogen for cell growth and antibiotic synthesis. Secondary metabolite formation by microorganisms has frequently been reported to occur principally during the stationary phase (idiophase), and numerous examples of this phenomenon are described in the literature (55, 56). However, it is also evident that the synthesis of such compounds may parallel the growth
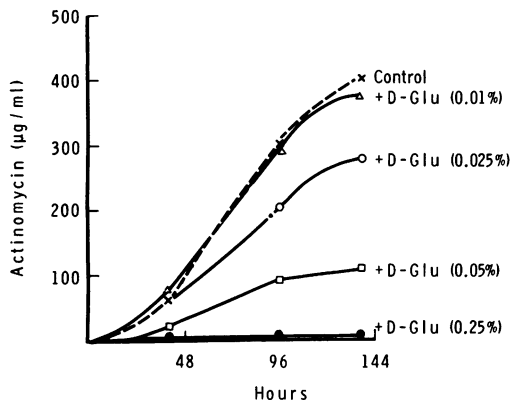

FIG. 9. *Repression of actinomycin formation by D-glucose. D-Glucose, at varying concentrations, was added at zero time to the 4% D-fructose-L-glutamic acid (15 mM)-L-histidine (5 mM)-basal mineral salts medium. Incubation was carried out for 137 h at 30°C. Symbols:* ×, *control; D-glucose:* △, *0.01%;* ○, *0.025%;* □, *0.05%;* ●, *0.25%.*
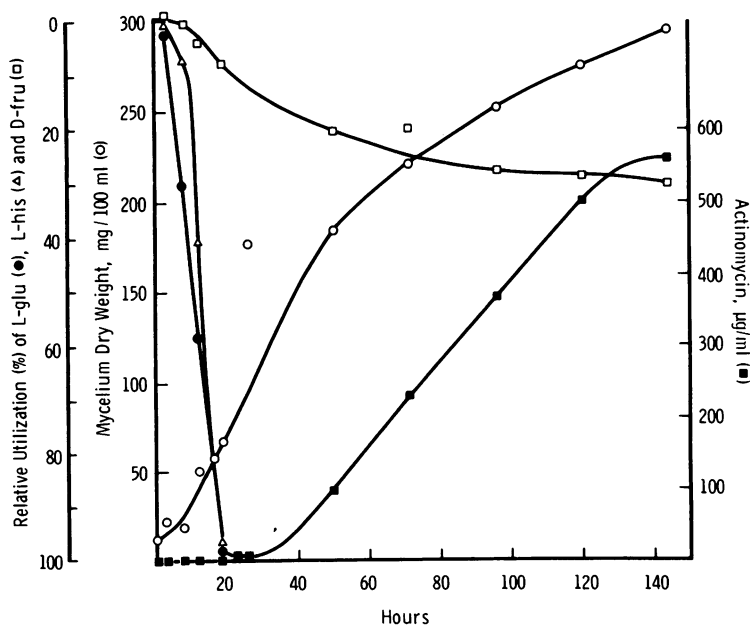

FIG. 10. *Dynamics of actinomycin fermentation: biochemical analyses of organic substrate utilization during growth and antibiotic synthesis by S. parvulus. The organism was cultivated in 4% D-fructose-L-glutamic acid (15 mM)-L-histidine (5 mM)-mineral salts medium for 144 h. Symbols:* ●, *L-Glutamic acid;* △, *L-histidine;* □, *D-fructose;* ○, *mycelium dry weight;* ■, *actinomycin.*

of the producing organism (3, 24), or that they may be formed during continuous cultivation of an organism (6, 23). As pointed out by Haavik (20, 22), the production of an antibiotic, after active growth of a culture, may depend on the particular nutritional conditions employed, or could arise as a consequence of the metabolic activities of the producing organism (e.g., formation of organic acids). Antibiotic synthesis may take place simultaneously with the cell's growth and may exert its effects throughout the growth cycle (23, 25, 29).

The repression of antibiotic formation by glucose in *S. parvulus* is similar to our previous experience with *S. antibioticus* (16). As the pH of the control and glucose-containing media increased from 7.1 to 8.0, the reduced antibiotic titer did not appear to be due to the lowering of pH through the production of organic acids (20, 22). The effect of glucose upon actinomycin production noted here may represent a repression of "actinomycin synthetase" formation (16).

Cations have been found to be important factors in the biosynthesis of antibiotics and other metabolic products by microorganisms (55). While we have not examined the role of the microelements systematically, in relation to the cell's growth and antibiotic production, nor rigorously purified the medium constituents, certain tentative conclusions can be drawn from the trace metal experiments (Fig. 5 to 8). The optimal yields of actinomycin were obtained in media containing Mg^{2+}, 4.88 $\mu g/ml$; Fe^{2+}, 8 to 10 $\mu g/ml$; Zn^{2+}, 4.6 to 13.7 $\mu g/ml$; Ca^{2+}, 7.5 to 14.6 $\mu g/ml$; and Co^{2+}, 0.003 to 0.006 $\mu g/ml$. Poor production was clearly observed if Fe^{2+} and Mg^{2+} (and Zn^{2+}) were omitted from the medium; however, such effects may be secondary to an initial influence on the growth of the producing culture. Additional studies are clearly required to provide insight into these relationships.

S. parvulus produces actinomycin D almost exclusively during antibiotic formation. The use of *S. parvulus* in contrast to *S. antibioticus* or *S. chrysomallus* for directed biosynthetic studies has certain advantages as the latter organisms normally produce five and three actinomycin components, respectively (29, 40). When an amino acid analogue is supplied to either of these cultures, the actinomycin mixtures formed are too complex to be characterized adequately. By contrast, a recent investigation with *S. parvulus*, employing cis- or trans-4-methyl-DL-proline, revealed that only two novel actinomycins (in major amounts) were synthesized in addition to actinomycin D (unpublished data).

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