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Mineral Nutrition of *Streptomyces kanamyceticus* for Kanamycin Formation

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Kanamycin production by *Streptomyces kanamyceticus* ATCC 12853 requires magnesium sulfate and potassium phosphate at concentrations of 0.4 and 1.0 g per liter, respectively. The optimal concentrations of Fe and Zn for production of kanamycin are 0.25 and 0.575 μg/ml, respectively, whereas Mo at 0.04 μg/ml allows maximal cellular growth and antibiotic synthesis. Mn and Ca are without any effect. Cu, Co, Ni, and V have inhibitory effect on growth of the organism as well as on kanamycin formation.

Microorganisms require some specific minerals for their growth and metabolic activities. This requirement for metals varies with the type of the organism as well with the nature of basal medium used. A considerable number of studies have been made on the requirement of metals for growth of different microbes, but there are few detailed investigations of the requirement for metals for actinomycetes during antibiotic biosynthesis (1, 2, 4, 5, 6, 8–12, 14, 16, 17, 21–25). Results obtained by different authors are sometimes contradictory, probably because of the difficulty of preparing a metal-free medium and also because requirements of metals for antibiotic production vary with the basal medium.

Umezawa et al. reported on the use of K, Mg, Na, Ca, and phosphate in complex media for kanamycin production by *Streptomyces kanamyceticus* K2J (23). For a study of the absolute requirement for metals, synthetic medium is usually selected, as complex media may contain considerable amounts of trace elements as contaminants. In the present work, an extensive study has been made on the mineral requirements of *S. kanamyceticus* for growth and kanamycin production in a synthetic medium.

MATERIALS AND METHODS

The culture of *S. kanamyceticus* was maintained on a maltose-sodium nitrate-minerals agar slant (14, 15) at 28 C and was subcultured at monthly intervals. The effects of different minerals were studied in the medium consisting of: galactose, 20.0 g; sodium nitrate, 5.1 g; K_2HPO_4, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeSO_4 \cdot 7H_2O$, 0.0025 g; $ZnSO_4 \cdot 7H_2O$, 0.0005 g; water, 1,000 ml; pH 7.5 ± 0.1. Triple glass-distilled water was used throughout the experiments.

In a typical experiment, the nutrient being tested was first omitted from and then added to the basal medium in graded doses in separate flasks to determine the optimal concentration. In each subsequent experiment, the composition of the basal medium was so altered as to include an optimal amount of an element from the previous study. Chemicals for the medium were of analytical reagent grade and were obtained from E. Merck. Sugar, sodium nitrate, and dipotassium hydrogen phosphate were further made free from trace elements by two different purification techniques: (i) the chelation method (13) and (ii) the adsorption method (18).

With the chelation method, the necessary amounts of galactose, sodium nitrate, and dipotassium hydrogen phosphate were each dissolved separately in 200 ml of triple-distilled water, and the resulting solutions were shaken twice with a mixture of 0.1 g of 8-hydroxyquinoline and 5 ml of chloroform in a separating funnel—first at pH 7.2 and then at pH 5.2 (13). After each extraction, the solution was washed three times with 5 ml and then once with 10 ml of chloroform to free the medium from traces of 8-hydroxyquinoline. Clean Pyrex glassware was used throughout the study. Solutions of galactose, sodium nitrate, phosphate, and a mixture of other minerals were sterilized separately. It was observed from preliminary studies that use of the chelation method of purification of the medium for the removal of trace element impurities resulted in low synthesis of kanamycin by the organism.

So another method of purification, viz., the adsorption method, was employed to make the solutions of sugar, sodium nitrate, and dipotassium hydrogen phosphate free from trace minerals (18).

The solutions of sugar, nitrogen source, and phosphate were each heated in an autoclave for 15 min, at 15 lb/in², pressure in the presence of excess precipitated calcium carbonate (15 g/liter), and filtered through Whatman no. 1 filter paper while hot. Removal of the trace element impurities was caused by the increased alkalinity, which causes a precipitation of the alkaline earth metal (Ca) simultaneously with the other undesired trace cationic elements as phosphates, hydroxides, carbonates, or basic carbonates.

The calcium precipitate itself serves as a gatherer or adsorbent. The filtered solutions of sugar, nitrate, phosphate and the mineral solution were sterilized separately and mixed in required amounts just prior to inoculation. After the medium had been dispensed in 30-ml volumes in 100-ml Erlenmeyer flasks, its pH was adjusted to 7.5 ± 0.1.

The inoculum was developed in Lepage broth (3) for 48 h. The 48-h-old cells of *S. kanamyceticus* in Lepage broth were centrifuged, washed twice with sterile water, and then suspended in sterile water. A 0.5-ml portion of this suspension was used to inoculate each flask. Flasks were incubated at 28 C on a rotary shaker (250 rpm, eccentricity 1.27 cm) for 7 days. Occasional checking of the flasks to drop adhering cells into the medium was necessary during the first 48 h of shaking. Incubation temperature was 28 C.

Determination of kanamycin potency. The filtered broth was diluted with potassium phosphate buffer (pH 8), and the estimation of kanamycin was made by a modified cup-plate method, with *Bacillus subtilis* (strain B_3) as the test organism. Kanamycin sulfate (Kantrex; Bristol Laboratories, Syracuse, N.Y.) was used as the standard, and the results are expressed in terms of micrograms of antibiotic per milliliter.

Determination of growth. The mycelium was separated from the culture broth by means of suction filtration on a sintered-glass funnel (G-1) through a Whatman no. 1 filter paper disk (3.0 cm) which had previously been dried for 24 h at 70 ± 5 C. After filtration, the paper disks were dried again at 70 ± 5 C to constant weight and weighed. To check the variation of weight of filter paper, a blank was also prepared in each set.

A glass-electrode pH meter was used for pH measurement.

RESULTS AND DISCUSSION

For studying the effects of $MgSO_4 \cdot 7H_2O$, K_2HPO_4, NaCl, and KCl on kanamycin production, rigorous purification of the medium was not done, as these were tested at higher concentrations. The results are shown in Tables 1 to 3. The optimal concentrations of $MgSO_4 \cdot 7H_2O$ and K_2HPO_4 for kanamycin formation in the synthetic medium are 0.4 g and 1.0 g per liter, respectively (Fig. 1 and Tables 1 and 2).

NaCl and KCl were without any effect (Table 3). Higher concentration of phosphate interfered with kanamycin synthesis. A similar observation was made by Majumdar and Majumdar in neomycin biosynthesis (14).

The effects of the elements Fe, Zn, and Ca on growth of *S. kanamyceticus* for kanamycin synthesis were first studied in the synthetic medium purified by the chelation method. It was observed that the elements iron and zinc are required at concentrations of 0.5 and 0.115 μg/ml, respectively, for maximal antibiotic yield (data not shown). Calcium is without any effect on kanamycin synthesis. Iron was essentially required for both growth and antibiotic formation, although its optimal concentrations for growth and antibiotic synthesis were different. Zinc is essential for antibiotic formation as no kanamycin was synthesized in the absence of zinc.

But the most striking observation was that the medium purified by the chelation method

TABLE 1. *Effect of $MgSO_4 \cdot 7H_2O$ on kanamycin formation by S. kanamyceticus*[a]

$MgSO_4 \cdot 7H_2O$ concn (g/liter)	Kanamycin (μg/ml) at day:		
	3	5	7
0.1	35.5	89.0	120
0.2	47.0	100.0	140
0.4	89.5	122.7	175
0.5	89.0	120.7	175
0.8	35.0	89.0	120

[a] Medium (30 ml per 100-ml Erlenmeyer flask) contained basal mineral salts, 2% galactose, and 0.51% sodium nitrate and was incubated on a rotary shaker at 250 rpm at 28 C.

TABLE 2. *Effect of K_2HPO_4 on kanamycin formation by S. kanamyceticus*[a]

K_2HPO_4 concn (g/liter)	Kanamycin (μg/ml) at day:		
	3	5	7
0	1.6	4.0	20
0.5	43.0	60.0	122
1.0	89.0	122.0	175
1.5	35.0	66.5	120
2.0	20.0	56.5	100

[a] Cultivation conditions were the same as described in the footnote to Table 1.

TABLE 3. *Effect of salts like KCl and NaCl on kanamycin formation by S. kanamyceticus*[a]

Salt	Concn (g/100 ml)	Kanamycin (μg/ml) at day:		
		3	5	7
KCl	0	88.0	120	175
	0.10	88.5	120	175
	0.25	85.0	100	150
	0.40	79.0	100	150
	0.50	75.0	90	120
NaCl	0	89	122	175
	0.25	87	120	170
	0.50	68	97	127

[a] Cultivation conditions were as described in the footnote to Table 1.

supported poor production of kanamycin in the presence of added minerals like Fe, Zn, and Ca. There was also lower synthesis of cellular materials. The chelating agent might have toxic effect on growth of the organism as well as on antibiotic biosynthesis. The toxic effect of chelating agents on cellular growth was also reported by Donald et al. (7). The effects of iron, zinc, calcium, and other minerals were, therefore, studied in a synthetic medium purified by the adsorption method. The results are shown in Tables 4 to 7 and graphically represented in Fig. 2 and 3.

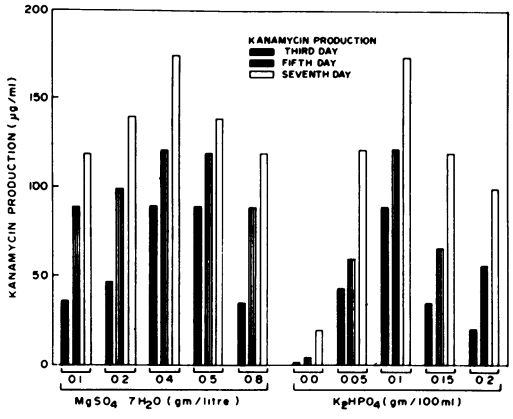

FIG. 1. *Effect of $MgSO_4 \cdot 7H_2O$ and K_2HPO_4 on kanamycin formation.*

It appears from the results that the elements Fe, Zn, and Mo are required at concentrations of 0.25, 0.575, and 0.04 μl/ml, respectively, whereas Ca is without any stimulatory effect on kanamycin synthesis; rather, at higher concentrations, it exerted some inhibitory effect (Table 7 and Fig. 3b). Mo had slight stimulatory effect on both growth of the organism and antibiotic formation (Table 6 and Fig. 3a). Cu, Mn, Co, Ni, and V were also studied for their effects on growth and antibiotic formation (data not shown). Cu, Co, and Ni were found to be inhibitory to the growth of the organism, and the antibiotic yield was also lowered in each case. Mn and V were without any pronounced effect either on cellular growth or antibiotic formation.

A careful observation of the individual roles played by different metal ions reveals that iron has an important role both in cellular growth and in kanamycin biosynthesis (Table 4 and Fig. 2a). It is interesting to note that cellular growth (though poor) is possible even in the

TABLE 4. *Effect of iron (added as $FeSO_4 \cdot 7H_2O$) on growth of S. kanamyceticus and kanamycin formation*[a]

Iron concn (μg/ml)	Day 3			Day 5			Day 7		
	pH	Growth (mg/100 ml)	Kanamycin (μg/ml)	pH	Growth (mg/100 ml)	Kanamycin (μg/ml)	pH	Growth (mg/100 ml)	Kanamycin (μg/ml)
0	7.5	37	1.4	8.2	170	5	8.2	200	10.0
0.25	7.7	291	51.0	8.4	360	91	8.6	357	120.0
0.50	7.9	290	27.0	8.4	360	70	8.6	360	95.0
1.0	8.0	249	23.0	8.3	357	44	8.5	351	55.0
1.5	7.7	248	21.0	8.2	310	32	8.5	310	52.0
2.0	7.7	197	15.0	8.0	240	25	8.4	240	51.0

[a] Cultivation conditions were as described in the footnote to Table 1.

TABLE 5. *Effect of zinc (added as $ZnSO_4 \cdot 7H_2O$) on growth of S. kanamyceticus and kanamycin formation*[a]

Zinc concn (μg/ml)	Day 3			Day 5			Day 7		
	pH	Growth (mg/100 ml)	Kanamycin (μg/ml)	pH	Growth (mg/100 ml)	Kanamycin (μg/ml)	pH	Growth (mg/100 ml)	Kanamycin (μg/ml)
0	8.2	191	0	8.4	231	0	8.5	285	0
0.0115	8.2	195	0	8.4	285	0	8.5	287	2.5
0.115	8.2	319	50	8.6	371	90	8.7	366	125
0.23	8.2	319	81	8.6	365	110	8.7	359	155
0.575	8.2	314	95	8.7	361	140	8.7	350	180
1.115	8.2	317	85	8.7	360	110	8.7	353	150

[a] Cultivation conditions were as described in the footnote to Table 1.

TABLE 6. *Effect of molybdenum (added as $Na_2MoO_4 \cdot 2H_2O$) on growth of S. kanamyceticus and kanamycin formation*[a]

Molybdenum concn (μg/ml)	Day 3			Day 5			Day 7		
	pH	Growth (mg/100 ml)	Kanamycin (μg/ml)	pH	Growth (mg/100 ml)	Kanamycin (μg/ml)	pH	Growth (mg/100 ml)	Kanamycin (μg/ml)
0	8.5	319	91	8.6	360	130	8.7	438	180
0.02	8.5	340	91	8.6	370	132	8.7	501	184
0.04	8.5	325	95	8.6	371	135	8.7	510	195
0.1	8.4	317	92	8.5	374	130	8.5	490	171
0.4	8.4	301	61	8.5	365	85	8.5	395	113
1.0	8.4	303	54	8.5	371	71	8.5	376	81

[a] Cultivation conditions were as described in the footnote to Table 1.

TABLE 7. *Effect of calcium (added as $CaCl_2 \cdot 2H_2O$) on growth of S. kanamyceticus and kanamycin formation*[a]

Calcium concn (μg/ml)	Day 3			Day 5			Day 7		
	pH	Growth (mg/100 ml)	Kanamycin (μg/ml)	pH	Growth (mg/100 ml)	Kanamycin (μg/ml)	pH	Growth (mg/100 ml)	Kanamycin (μg/ml)
0	8.3	301	97	8.5	467	138	8.8	457	195
5.4	8.3	327	91	8.5	469	133	8.8	510	187
10.8	8.3	321	81	8.5	471	110	8.7	433	150
21.6	8.2	221	55	7.4	275	71	8.0	305	50
54.0	8.2	222	41	7.2	255	33	7.0	226	21
108.0	8.2	209	12	7.0	241	6	7.0	223	5

[a] Cultivation conditions were as described in the footnote to Table 1.

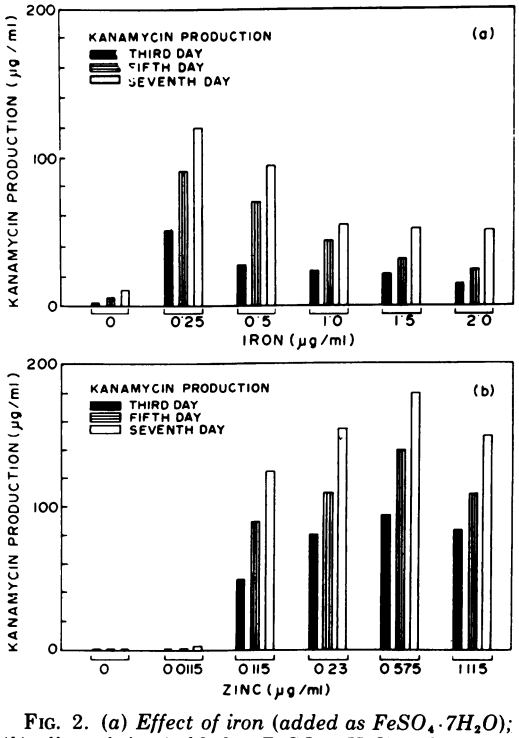

FIG. 2. (*a*) *Effect of iron (added as $FeSO_4 \cdot 7H_2O$);* (*b*) *effect of zinc (added as $ZnSO_4 \cdot 7H_2O$) on kanamycin formation.*

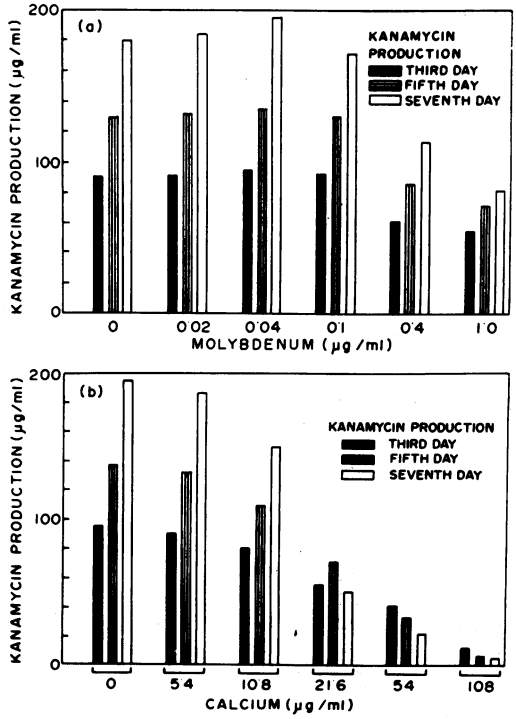

FIG. 3. (*a*) *Effect of molybdenum (added as $Na_2MoO_4 \cdot 2H_2O$);* (*b*) *effect of calcium (added as $CaCl_2 \cdot 2H_2O$) on kanamycin formation.*

complete absence of iron. This might be due to two reasons: (i) the medium used could not be made perfectly free from iron, or (ii) some other bivalent cations exerted sparing effect for iron.

Zinc is another important trace element for the synthesis of kanamycin by the organism *S. kanamyceticus*. No kanamycin was produced in the absence or at low concentrations of zinc (Fig. 2b), although there was some considerable growth of the organism (Table 5). As a stimulator of kanamycin synthesis, zinc might be involved in the enzyme system utilized at some steps of kanamycin biosynthesis. The optimal concentration of zinc for kanamycin formation was five times the concentration required for optimal growth (Table 5). Similar observation with respect to iron on streptomycin biosynthesis was reported by Chesters and Rollinson (5).

Both Mo and Ca were found to promote cellular growth; Mo also showed slight stimulation of antibiotic synthesis.

As a result of the present study a suitable synthetic medium was selected with the following composition: galactose, 20.0 g; $NaNO_3$, 5.1 g; K_2HPO_4, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.4 g; $FeSO_4 \cdot 7H_2O$, 0.00125 g; $ZnSO_4 \cdot 7H_2O$, 0.0025 g; $Na_2MoO_4 \cdot 2H_2O$, 0.0001 g; water, 1,000 ml; pH 7.5 $\pm$ 0.1. This medium produced 195 μg of kanamycin/ml.

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LITERATURE CITED

1. Acker, R. F., and H. A. Lechevalier. 1954. Some nutritional requirements of *Streptomyces griseus* 3570 for growth and candicidin production. Appl. Microbiol. **2**:152-157.
2. Bailey, J., and C. J. Cavellito. 1943. Production of citrinin. J. Bacteriol. **45**:30-31.
3. Basak, K., and S. K. Majumdar. 1973. Utilization of carbon and nitrogen sources by *Streptomyces kanamyceticus* for kanamycin production. Antimicrob. Agents Chemother. **4**:6-10.
4. Bonner, J., and J. E. Varner. 1965. Mineral metabolism, p. 438-461. *In* Plant biochemistry. Academic Press Inc., New York.
5. Chesters, C. G. C., and G. N. Rollinson. 1951. Trace elements and streptomycin production. J. Gen. Microbiol. **5**:559-565.
6. Cochrane, V. W. 1958. Inorganic nutrition and metabolism, p. 300-317. *In* Physiology of fungi. John Wiley and Sons Ltd., New York.
7. Donald, C., B. I. Passey, and R. H. Swaby. 1952. A comparison of methods for removing trace metals from microbiological media. J. Gen. Microbiol. **7**:211-220.
8. Gallicchio, V., and D. Gottlieb. 1958. The biosynthesis of chloramphenicol. III. Effects of micronutrients on synthesis. Mycologia **50**:490-496.
9. Heim, A. H., and H. A. Lechevalier. 1956. Effect of iron, zinc, manganese and calcium on the growth of various strains of *Streptomyces*. Mycologia **48**:628-636.
10. Jansen, E. F., and D. J. Hirschmann. 1944. Subtilin—an antibacterial product of *Bacillus subtilis* culturing conditions and properties. Arch. Biochem. **4**:297-309.
11. Katz, E., P. Pienta, and A. Sivak. 1958. The role of nutrition in the synthesis of actinomycin. Appl. Microbiol. **6**:236-241.
12. Koffler, H., S. G. Knight, and W. C. Frazier. 1947. The effect of certain mineral elements on the production of penicillin in shake flasks. J. Bacteriol. **53**:115-123.
13. Majumdar, S. K., and S. K. Bose. 1960. Trace element requirements of *Bacillus subtilis* for mycobacillin formation. J. Bacteriol. **79**:564-565.
14. Majumdar, M. K., and S. K. Majumdar. 1965. Effect of minerals on neomycin production by *Streptomyces fradiae*. Appl. Microbiol. **13**:190-193.
15. Majumdar, M. K., and S. K. Majumdar. 1967. Utilization of carbon and nitrogen-containing compounds for neomycin production by *Streptomyces fradiae*. Appl. Microbiol. **15**:744-749.
16. Nickerson, W. J., and R. R. Mohan. 1953. Nutrition and metabolism of *Streptomyces*. 6th Int. Congr. Microbiol., Rome, p. 137-147.
17. Reynolds, D. M., and S. A. Waksman. 1948. Grisein, an antibiotic produced by certain strains of *Streptomyces griseus*. J. Bacteriol. **55**:739-752.
18. Steinberg, R. A. 1919. A study of some factors in the chemical stimulation of the growth of *Aspergillus niger*. Am. J. Bot. **6**:330-372.
19. Steinberg, R. A. 1950. Trace element impurities in nutrient solutions for fungi. Arch. Biochem. **18**:111-116.
20. Thornberry, H. H. 1946. Nutrient requirements of an antibiotic soil fungus, *Streptomyces griseus* (Krainsky) Waksman & Henrici. Phytopathology **36**:412.
21. Thornberry, H. H. 1948. The role of minerals in production of streptomycin by *Streptomyces griseus*. Phytopathology **38**:26.
22. Thornberry, H. H., and H. W. Anderson. 1948. Synthetic medium for *Streptomyces griseus* and the production of streptomycin. Arch. Biochem. **16**:389-397.
23. Umezawa, H., M. Ueda, K. Maeda, K. Yagishita, S. Kondo, Y. Okami, R. Utahara, Y. Osato, K. Nitta, and T. Takeuchi. 1957. Production and isolation of a new antibiotic kanamycin. J. Antibiot. **10**:181-188.
24. Waksman, S. A. 1959. The actinomycetes, vol. 1, p. 138. The Williams and Wilkins Co., Baltimore.
25. Webley, D. M. 1960. The effect of deficiency of iron, zinc and manganese on the growth and morphology of *Nocardia opaca*. J. Gen. Microbiol. **23**:87-92.