STUDIES ON THE UTILIZATION OF LIPIDS BY STREPTOMYCES GRISEUS[1]

D. PERLMAN AND G. H. WAGMAN

Squibb Institute for Medical Research, New Brunswick, New Jersey

Received for publication August 3, 1951

In studies on the production of streptomycins by *Streptomyces griseus*, Schatz *et al.* (1944) and Waksman *et al.* (1946, 1948) used media containing glucose, meat extract, peptone, and sodium chloride. While the meat extract and peptone components of the media were replaced in later experiments by such materials as soybean meal (Rake and Donovick, 1946), cornsteep liquor, asparagus butt juice, and hydrolyzed proteins of various types (Bennett, 1947), glucose was retained as the carbohydrate and main energy source. Hubbard and Thornberry (1946), Saunders and Sylvester (1947), and Dulaney (1949) in studying the composition of synthetic media suitable for the growth of *S. griseus* cultures and the production of streptomycin, noted that other carbohydrates could be substituted for the glucose component of these media without significantly changing the production of streptomycin. However, these reports indicated that little advantage would be gained from such substitution. While the above-mentioned studies were completed on a laboratory scale, it appears that in large scale operations media containing glucose have also been used (Porter, 1946).

Several years ago in a patent granted to F. J. Rudert (1945), examples were given of the substitution of lipids such as corn oil for the glucose or sucrose component of media to be fermented by *Eremothecium ashbyii*. Replacements of this type resulted in equal or increased yields of riboflavin by this organism, and examples in other patents confirmed this observation with *E. ashbyii* (Phelps, 1949) and suggested that this substitution could also be made in media on which the related organism, *Ashbya gossypii*, was grown (Tanner *et al.*, 1948).

While the literature contains many references to the use of oils in fermentations as antifoaming agents, there are few reports on the use of oils as energy sources, and apparently lipid metabolism has not received much attention. Pratt and Dufrenoy (1949), Koffler and Goldschmidt (1949), and Goldschmidt and Koffler (1950) observed that the addition of many vegetable and animal oils or of fatty acids to media resulted in increased penicillin production (submerged culture process), but it is not certain from their data whether these substances acted as energy sources. Holtman (1945) considered the possibility that corn oil might act as an energy source in his experiments on the production of penicillin in surface culture although he did not confirm this suggestion by further studies. The experiments to be discussed hereafter indicate that *S. griseus* and other actinomycetes utilize many lipids including those found in animal and vegetable oils as energy sources, and that substitution of lipids for the glucose

[1] Presented in part before the Fermentation Section, Agriculture and Food Division of the American Chemical Society, April 12, 1950.

or other carbohydrates used in media for the cultivation of these actinomycetes does not result in reduction in antibiotic production.

EXPERIMENTAL METHODS

All of the experiments to be discussed were done on a laboratory scale. Shaken flask fermentations (100 ml of medium in a 5000 ml Erlenmeyer flask) inoculated with vegetative growth of *S. griseus* (Waksman strain 4) were placed on a reciprocating shaker located in a 25 C constant temperature room as used elsewhere (Rake and Donovick, 1946). Samples were removed from replicate flasks during the incubation period, pooled, and analyzed for a number of metabolic products.

Streptomycin content was determined by the tube-dilution test described by Donovick *et al.* (1945) and by a modification of the chemical assay described by Eisenman and Bricker (1949). Since both streptomycin and mannosidostreptomycin are produced by this culture when grown under submerged culture conditions, the chemical assay was used (where indicated) as an estimate of the total weight concentrations of streptomycin and mannosidostreptomycin. The fermentation samples were treated with acid (Rake *et al.*, 1949) before streptomycin assay in order to free the antibiotic adsorbed on the mycelium (Perlman and Langlykke, 1949).

Aliquots of the samples were examined for the presence of residual sugar by the Shaffer-Somogyi (1933) method (using reagent 50), acetic acid by a modification of the method of Virtanen and Pulkki (1928), lactic acid by the method of Barker and Summerson (1941), and residual lipid by a modification of Bloor's method (1928) using a sample of refined soybean oil (iodine number: 125) as a standard.

Other strains of *S. griseus* were used in a number of other experiments, but only the data obtained with the Waksman strain 4 are presented here. This culture has been studied in a number of laboratories, and its characteristics are well known.

RESULTS

Chemical changes occurring during the fermentation of soybean meal-lipid media. Preliminary experiments revealed that this organism would grow well on a medium containing 15 g of soybean meal (expeller process) and 20 g of glucose per liter and that the glucose could be replaced by a number of lipids including corn, soybean, lard, cottonseed, linseed, olive, and peanut oils. Several studies investigating some of the chemical changes occurring during the fermentations are summarized in figures 1 and 2. Figure 1 illustrates the course of fermentation of a 2 per cent glucose medium and a calorically equivalent soybean oil medium. Slightly more acetic acid was found in fermentations where soybean oil was the energy source than in fermentations where glucose was the energy source.

The course of fermentation of a 4 per cent glucose medium and a 2 per cent soybean oil medium is illustrated in figure 2. Increasing the glucose content of the medium resulted in an increase in the quantity of acetic acid produced, as did an increase in the soybean oil content. The rate of utilization of glucose was sig-

nificantly higher than the rate of utilization of the soybean oil, resulting in rapid disappearance from the medium and coincident autolysis and fragmentation of the cellular structure and production of ammonia. This slow utilization of the

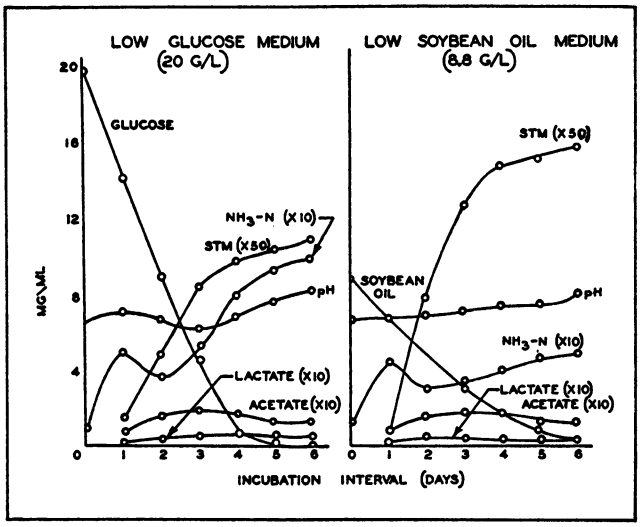

Figure 1. Chemical changes occurring during fermentation of a low energy source soybean meal medium.

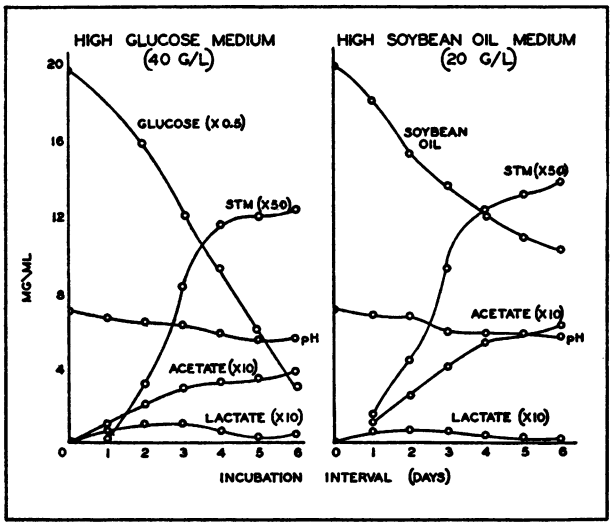

Figure 2. Chemical changes occurring during fermentation of a high energy source soybean meal medium.

soybean oil and the resulting delayed autolysis of the culture may account for the relatively stable pH of the fermentations of soybean oil containing media. While lactic acid was found in both fermentations, it apparently does not con-

stitute a major dissimilation product of either soybean oil or glucose by this actinomycete when grown under these conditions.

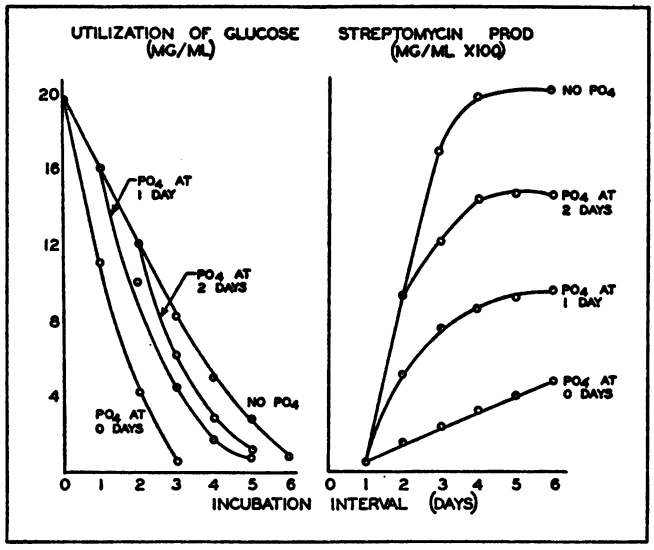

Figure 3. Effect of addition of inorganic phosphate to a soybean meal—glucose medium on streptomycin production and glucose utilization. (1 g $K_2HPO_4 \cdot 3H_2O$ added per liter.)

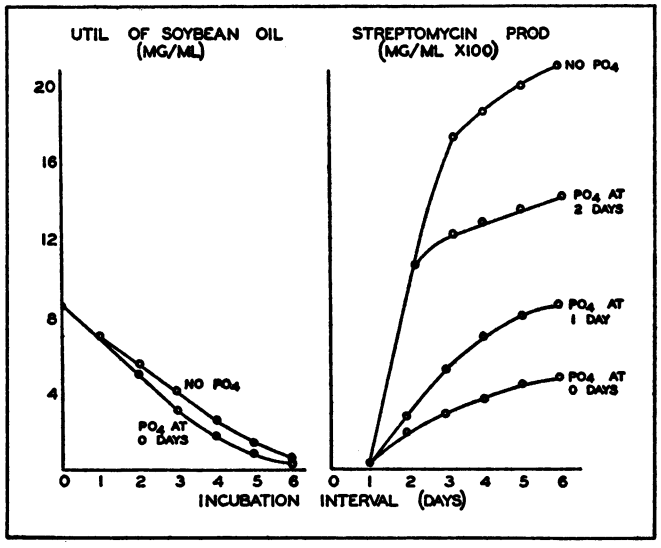

Figure 4. Effect of addition of inorganic phosphate to a soybean meal—soybean oil medium on streptomycin production and lipid utilization. (1 g $K_2HPO_4 \cdot 3H_2O$ added per liter.)

In figure 3 are presented some of the results of adding inorganic phosphate to the streptomycin fermentation of the soybean meal-glucose medium. In con-

firmation of earlier observations on the fermentation of a meat extract-glucose medium (Dulaney and Perlman, 1947), the yield of streptomycin is reduced with an associated increase in the rate of glucose metabolism. As the data of figure 4 show, the reduction in streptomycin yield is probably not directly related to the rate of utilization of the energy source, since the addition of inorganic phosphate to the soybean meal-soybean oil medium also results in reduced streptomycin yields without, however, any effect on the rate of metabolism of the oil. The inhibition of streptomycin production by phosphate may explain the requirement for calcium carbonate in media which incorporate cornsteep water which contributes a high proportion of phosphate (Fortune *et al.*, 1950).

TABLE 1

Streptomycin production on soybean meal media containing vegetable oils as energy sources

ENERGY SOURCE*	G/L	STREPTOMYCIN PRODUCTION (UNITS/ML)			
		3 days	5 days	7 days	
		Bioassay	Bioassay	Bioassay	Chemical assay
None	—	64	78	67	105
Glucose	20.0	163	225	223	298
	10.0	157	209	206	242
Soybean oil	20.0	151	173	196	380
	8.8	188	381	279	415
	4.4	279	331	249	422
Cottonseed oil	20.0	185	173	181	287
	8.8	207	381	323	400
	4.4	288	353	281	380
Coconut oil	20.0	100	105	92	315
	8.8	235	323	341	440
	4.4	334	288	266	340

* Basal medium (g/L): soybean meal, 15 g; water to 1 liter.

For the purpose of studying the oxidation of lipids by washed cells of *S. griseus*, cells from a 48-hr fermentation were washed with and suspended in sterile distilled water and then replaced on the shaker for a 24-hr period. This aging period was employed in order to reduce the endogenous respiration. Cells grown on media containing lard oil appeared to be more active metabolically than cells grown on media containing glucose (Q_{O_2} on glucose media was 50 as compared with 27). Addition of inorganic phosphate speeded the metabolism of glucose and glycerol by both types of cells (as measured by oxygen demand) but did not affect the rate of metabolism of stearate, acetate, or lard oil by these cells.

Effect of substitution of lipids for glucose on antibiotic production. The foregoing discussion summarizes some of the observations made of the chemical changes occurring when *S. griseus* is grown on media in which lipids are used to replace the

TABLE 2

Streptomycin production on soybean meal media containing fatty acids and pure glycerides as energy sources

ENERGY SOURCE*	G/L	STREPTOMYCIN PRODUCTION (UNITS/ML)		
		4 days	6 days	
		Bioassay	Bioassay	Chemical assay
None	—	70	72	155
Glucose	20.0	135	196	285
	10.0	146	183	276
Stearic acid	20.0	91	137	203
	8.8	121	178	247
	4.4	134	201	281
Tristearin	20.0	67	101	189
	8.8	101	163	231
	4.4	122	189	257
Lauric acid	20.0			
	8.8		No growth	
	4.4			
Trilaurin	20.0	43	79	131
	8.8	71	131	208
	4.4	76	157	224

* Basal medium (g/L): soybean meal, 15 g; water to 1 liter.

TABLE 3

Streptomycin production on soybean meal media containing lard oil and glucose mixtures as energy sources

MEDIUM COMPOSITION		STREPTOMYCIN PRODUCTION (UNITS/ML)		
		4 days	6 days	
Glucose, G/L	Lard oil, G/L	Bioassay	Bioassay	Chemical assay
20	—	96	156	330
15	—	200	190	310
10	—	166	182	215
15	2.2	91	151	230
10	10.0	96	97	210
10	4.4	96	172	262
5	15.0	159	175	260
5	6.6	125	137	245
—	20.0	96	162	195
—	8.8	96	264	350
—	6.6	114	285	395
—	4.4	193	183	250

* Other medium ingredients (g/L): soybean meal, 15 g, water to 1 liter.

carbohydrate component. The data collected indicate that the metabolism of the actinomycete is not changed markedly when such a substitution is made. A number of experiments studying the effect on antibiotic production of this substitution are summarized in tables 1 to 5. Preliminary studies had indicated that highest antibiotic titers were usually obtained under laboratory conditions when the actinomycete was grown on a medium containing 15 g of soybean meal (expeller process) and 20 g of glucose for a 5- to 7-day period, and all experimental media are compared here with this "standard medium."

The data summarized in table 1 indicate some of the results obtained when soybean oil, cottonseed oil, and coconut oil are substituted for the glucose component of the soybean meal medium. As the culture used in these experiments produces both streptomycin and mannosidostreptomycin, chemical assays gave higher values than those indicated by the microbiological assays. However, there is no definite suggestion that the use of oils in media leads to change in the relative proportions of the streptomycin produced. The data summarized in table 1 suggest that highest antibiotic activity is obtained when the replacement is made on a caloric basis rather than a weight basis. This agrees with the hypothesis that the actinomycete metabolizes the oil to carbon dioxide, as was suggested by the metabolic studies mentioned before. Consideration of the possible metabolic mechanisms used in this conversion suggests that the organism is able to hydrolyze the oil to fatty acids although actinomycetes do not appear to be strongly lipolytic organisms. Esterification of the fatty acids with glycerol does not affect the utilization of stearic acid (as indicated by streptomycin production) but reduces the toxicity of lauric acid as indicated in table 2. This latter effect was anticipated as coconut oil, in spite of its high lauric acid content, supports good streptomycin production. Oleic and palmitic acids as well as their glycerides are also satisfactory substitutes for glucose.

A number of other vegetable and animal oils including corn, peanut, sesame, sperm, and lard oils and mutton tallow were studied as replacements for glucose, and the results obtained generally confirm the examples presented in table 1.

In most large-scale aerated fermentation operations the utilization of antifoaming agents has been found necessary. Animal and vegetable oils have been found useful for this purpose, and it seemed possible that the oils in question could act as energy sources as well as antifoaming agents. Hence, laboratory-scale fermentations were started, using mixtures of lard oil or soybean oil and glucose as energy sources. In these experiments, lower streptomycin yields were obtained than when either oil or glucose was used alone. Some of the data collected with glucose-lard oil mixtures are presented in table 3. Further laboratory studies suggested that in these fermentations the glucose was metabolized preferentially although the results were not clear-cut. On the other hand, inoculation of media containing oils as energy sources with inoculum grown on media containing glucose as energy source did not apparently affect the rate of streptomycin production when compared with similar fermentations inoculated with cells grown on oil-containing media. It is possible that there was a difference in the rates of utilization of these substrates during the early part of the fermentation period, but this was not noted during the relatively long induction phase.

TABLE 4

Streptomycin production on media containing ground seeds

MEDIUM COMPOSITION* (G/L)	STREPTOMYCIN PRODUCTION (UNITS/ML)		
	3 days	5 days	
	Bioassay	Bioassay	Chemical assay
Soybean meal, 15 g, glucose, 20 g	164	197	360
Ground soybean, 20 g	96	93	165
Ground soybean, 40 g	140	179	285
Ground peanuts, 20 g	123	161	265
Ground peanuts, 40 g	158	205	315

* All diluted to 1 liter with water.

TABLE 5

Streptomycin production on synthetic media containing lipids as energy source

ENERGY SOURCE*	G/L	STREPTOMYCIN PRODUCTION (UNITS/ML)	
		4 days	6 days
		Bioassay	Bioassay
None	—	27	53
Glucose	20.0	83	133
	10.0	149	133
	5.0	80	85
Lard oil	20.0	50	174
	8.8	80	180
	4.4	43	84
	2.2	64	90
Soybean oil	20.0	97	155
	8.8	97	191
	4.4	56	89
	2.2	50	71
Soybean oil Glucose	4.4 10.0	43	71
Soybean oil Glucose	10.0 10.0	38	75
Soybean oil Glucose	6.6 5.0	80	89

* Basal medium (g/L): L-proline, 15 g; NaCl, 5 g; K_2HPO_4, 2 g; $MgSO_4 \cdot 7H_2O$, 1 g; $CaCl_2$, 0.4 g; $FeSO_4 \cdot 7H_2O$, 0.02 g; $ZnSO_4 \cdot 7H_2O$, 0.01 g; water to 1 liter.

As soybeans and other oil-bearing seeds contain rather considerable quantities of oil, in some cases reaching 30 per cent of the weight of the seed, it occurred to us that perhaps ground seeds might be a suitable medium providing both nitrogen and energy sources for the growth of the actinomycete. The data summarized in table 4 tend to confirm this hypothesis, although the streptomycin yields obtained with media containing the ground seeds were slightly lower than those obtained with the soybean meal-glucose medium.

In all of the previous experiments soybean meal protein has been used as the source of available nitrogen in the media. It seemed advisable to study the use of lipids in media containing other nitrogen sources. The data summarized in table 5 indicate that the glucose component of a synthetic medium (Dulaney, 1948) may be replaced by lipids without reduction in streptomycin production. Also, it can be seen that the use of mixtures of lipid and glucose resulted in marked reduction in antibiotic production as had been previously noted with the soybean meal media. These experiments serve to emphasize that the nitrogenous components of the medium do not directly affect the replaceability of carbohydrates by fats or oils.

A few experiments have shown that other antibiotic-producing actinomycetes will grow well on media containing lipids as replacements for glucose or other carbohydrates. Highest antibiotic titers were obtained when this replacement was made on a caloric basis, and in some cases, e.g., production of neomycin and streptothricin, higher antibiotic production was obtained than when the carbohydrate-containing media were used. Other laboratory studies indicated that the production of chloramphenicol and aureomycin was not affected by this substitution.

ACKNOWLEDGMENT

We are indebted to Mr. D. N. Lapedes and his staff for the antibiotic assays.

SUMMARY

Lipids including animal and vegetable oils may be used as replacements for the glucose in media for the cultivation of antibiotic-producing *Streptomyces griseus* cultures without reduction in antibiotic production. Best results (as measured by antibiotic production) were obtained when this substitution was made on a caloric rather than on a weight basis.

REFERENCES

BARKER, S. B., AND SUMMERSON, W. H. 1941 The colorimetric determination of lactic acid in biological material. J. Biol. Chem., **138**, 535-554.

BENNETT, R. E. 1947 Nutrition of *Streptomyces griseus* in relation to streptomycin titer. J. Bact., **53**, 254.

BLOOR, W. R. 1928 The determination of small amounts of lipid in blood plasma. J. Biol. Chem., **77**, 53-73.

DONOVICK, R., HAMRE, D., KAVANAGH, F., AND RAKE, G. 1945 A broth dilution method of assaying streptothricin and streptomycin. J. Bact., **50**, 623-628.

DULANEY, E. L. 1948 Observations on *Streptomyces griseus*. II. Nitrogen sources for growth and streptomycin production. J. Bact., **56**, 305-314.

DULANEY, E. L. 1949 Observations on *Streptomyces griseus*. III. Carbon sources for growth and streptomycin production. Mycologia, **41**, 1–10.

DULANEY, E. L., AND PERLMAN, D. 1947 Observations on *Streptomyces griseus*. I. Chemical changes occurring during submerged streptomycin fermentations. Bull. Torrey Bot. Club, **74**, 504–511.

EISENMAN, W., AND BRICKER, C. E. 1949 Spectrophotometric method for determining streptomycin. Anal. Chem., **21**, 1507–1508.

FORTUNE, W. B., MCCORMICK, S. L., RHODEHAMEL, H. W., AND STEFANIAK, J. J. 1950 Antibiotics development. Ind. Eng. Chem., **42**, 191–198.

GOLDSCHMIDT, M. C., AND KOFFLER, H. 1950 Effect of surface active agents on penicillin yields. Ind. Eng. Chem., **42**, 1819–1823.

HOLTMAN, D. F. 1945 Increasing penicillin yields with corn oil. J. Bact., **49**, 313–314.

HUBBARD, C. V., AND THORNBERRY, H. H. 1946 Utilization of various carbohydrates by *Streptomyces griseus* for the production of streptomycin and growth. Trans. Ill. Acad. Sci., **39**, 57–64.

KOFFLER, H., AND GOLDSCHMIDT, M. C. 1949 The effect of certain antifoam agents on penicillin yields obtained during the submerged growth of *Penicillium chrysogenum* Q_{176}. Am. J. Botany, **36**, 811.

PERLMAN, D., AND LANGLYKKE, A. F. 1949 Methods for the extraction of streptomycin from fermentation media. Abst. 116th meeting, Am. Chem. Soc., 18A–19A.

PHELPS, A. S. 1949 Production of riboflavin. U. S. Patent 2,473,818.

PORTER, R. W. 1946 Streptomycin engineered into commercial production. Chem. and Met. Eng., **53**, (March) 94–98, 142–145.

PRATT, R., AND DUFRENOY, J. 1949 Antibiotics. J. B. Lippincott Company, Philadelphia, Pa.

RAKE, G., AND DONOVICK, R. 1946 Studies on the nutritional requirements of *Streptomyces griseus* for the formation of streptomycin. J. Bact., **52**, 223–226.

RAKE, G. W., KOERBER, W. L., AND DONOVICK, R. 1949 Antibiotic extraction from solids of *Actinomyces griseus* culture. U. S. Patent 2,461,922.

RUDERT, F. J. 1945 Riboflavin production by biochemical methods. U. S. Patent 2,374,503.

SAUNDERS, A., AND SYLVESTER, J. C. 1947 Synthetic media for the production of streptomycin. Abst. 112th meeting, Am. Chem. Soc., 9A–10A.

SCHATZ, A., BUGIE, E., AND WAKSMAN, S. A. 1944 Streptomycin, a substance exhibiting antibiotic activity against gram positive and gram negative bacteria. Proc. Soc. Exptl. Biol. Med., **55**, 66–69.

SHAFFER, P. A., AND SOMOGYI, M. 1933 Copper-iodometric reagents for sugar determination. J. Biol. Chem., **100**, 695–713.

TANNER, F. W., JR., WICKERHAM, L. J., AND VAN LANEN, J. M. 1948 Production of riboflavin. U. S. Patent 2,445,128.

VIRTANEN, A. I., AND PULKKI, L. 1928 The volatility of water-soluble organic substances with steam. J. Am. Chem. Soc., **50**, 3139–3151.

WAKSMAN, S. A., AND SCHATZ, A. 1948 Streptomycin and process of preparation. U. S. Patent 2,449,866.

WAKSMAN, S. A., SCHATZ, A., AND REILLY, H. C. 1946 Metabolism and the chemical nature of *Streptomyces griseus*. J. Bact., **51**, 753–759.