UTILIZATION OF SOME ORGANIC ACIDS BY *STREPTOMYCES GRISEUS* FOR STREPTOMYCIN PRODUCTION AND GROWTH

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Studies on the utilization of some organic acids by *Streptomyces griseus* (Krainsky) Waksman and Henrici, 1943 (33, 35) were undertaken to ascertain further information on the nutritional capacity of this microorganism in connection with streptomycin production. Such studies were favored by the availability of a synthetic medium for the production of streptomycin by this organism (28, 31). They also logically accompany the previous studies on the utilization of some sugars by this organism (17) since both glucose and lactate are important carbon constituents of the chemically defined medium (28, 31). At the time of initiating these studies in 1946, no reference was found relative to this organism utilizing organic acids[2] except malic acid (33).

Methods and Materials

The chemically defined basal medium used throughout these studies was a modification of the original synthetic medium (28) that permitted the addition of organic acids in varied amounts as substitutes for the lactic acid. It consisted of the following constituents of C. P. and Reagent grades:

Substance	Amount	Concentration (Final Molarity)
Glucose (Cerelose)	10. g.	0.0550
KH_2PO_4	2.38 g.	0.0176
$K_2HPO_4.3H_2O$	5.65 g.	0.0247
NH_4NO_3	4.00 g.	0.0500
$Mg\ SO_4.7H_2O$	1.00 g.	0.0040
$Zn\ SO_4.7H_2O$	0.0115 g.	0.00004
$Fe\ SO_4.7H_2O$	0.0111 g.	0.00004
$Cu\ SO_4$	0.0064 g.	0.00004
$Mn\ Cl.4H_2O$	0.0070 g.	0.00004
Distilled water (H-ion concentration adjusted to pH 7.0 ± 0.1 when necessary)	500 ml.	(1000 ml.)

In preparing the complete medium for each of the varied amounts of organic acids (racemic mixtures of the optically active acids), the necessary amounts of the organic acid in aqueous solution neutralized with NaOH to pH 7.0 was added to 75 ml. of the double strength basal medium. Distilled water was then added to make 150 ml. volume. Fifty ml. of this complete medium was then placed in 125 ml. Erlenmeyer flasks in triplicate and sterilized by autoclaving at 121°C. for 15 minutes. The organic acids used were those readily available.

The culture of *Streptomyces griseus*[3] used throughout these studies was a subculture of Waksman's strain No. 9 from No. 18-16 origi-

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[2] Referred to as acids rather than sodium salts of organic acids for brevity.

[3] Letter from S. A. Waksman to H. W. Anderson, September 1, 1944.

nally obtained from heavily manured field soil (26).

Other items of procedure involved the following methods and conditions:

(a) The inoculum, consisting of 3 or 4 drops of suspended fragments of mycelium, was applied to the surface of the medium (17).

(b) Growth conditions consisted of surface stationary culture (air surface of 0.5 cm^2/ml. of medium 2 cm. deep) and 26°C. for 10 days.

(c) Growth in 10 days was estimated qualitatively on an arbitrary scale of 0 to 10 and where adequate it was determined quantitatively as vacuum dried (80°C.) mycelial mat weight.

(d) The pH values were determined with a glass electrode Beckman pH meter.

(e) Streptomycin was determined in duplicate assays by the paper disc-plate method against *Bacillus subtilis* cultured at 30°C. (19). The fermented medium was diluted 1-4 in 0.1 molar phosphate buffer at pH 7.8 for the assays. Streptomycin values are reported as "S Units" of streptomycin per ml. (34). One "S Unit" of streptomycin is approximately one microgram of streptomycin free base (36).

(f) The effect of the nutritive acids on the assay of streptomycin was measured by mixing the acids individually at 0.1 molal final concentration with solutions of streptomycin six hours prior to assay in the usual procedure.

(g) The effect of these acids upon growth of the test organism in the assay plates was determined by applying them individually at 0.1 molal to the assay discs as in (f) above but without streptomycin.

Experiments and Results

For a preliminary survey on utilization, some organic acids were each evaluated at 0.05 molal in the basal synthetic medium. The results in table 1 indicate that some acids are not utilized at 0.05 molal. In fact, these acids either retard or inhibit the growth of *Streptomyces griseus*. These are butyric, a-hydroxy-e-benzoylamino caproic, monochloroacetic, crotonic, glycollic, formic, mandelic, oxalic, and tannic acids. Some other acids either do not affect the organism or affect it too slightly to yield noticeable comparative changes in pH, growth, or streptomycin production from the control of basal medium. These are tartaric (Na and Na and K salts) and tartronic acids. Other acids appear to be utilized. These are acetic, adipic, citric, glutaric, lactic (control), malic, malonic, and succinic acids.

Those acids that did not noticeably inhibit or retard growth at 0.05 molal were evaluated over a range of concentration. It was thought that results from this procedure should reveal whether utilization of organic acids might be related to concentration or to a suitable balance of these substances with other constituents in the medium. The results are given in charts (figs. 1 to 10) in order to show the relationship of three responses (pH, growth, and streptomycin production) to concentration of nutritive acid. These responses do not necessarily parallel each other or show any constant relationship with respect to either concentration or type of acid. The composite results in the charts indicate that these acids are utilized in some manner and to some extent by the organism in its metabolism under these conditions.

TABLE 1.—UTILIZATION OF ORGANIC ACIDS AT 0.05 MOLAL BY STREPTOMYCES GRISEUS IN STATIONARY CULTURE AT 26°C.

Acid	pH Initial	pH Final	Growth (10 days)	Mat weight (mg)	Streptomycin (units/ml)
Acetic	6.88	7.74	8	274	52
Adipic	6.83	6.92	10	323	48
Butyric	6.72	6.55	0	...[a]	0
a-hydroxy-e-benzoylamino caproic	6.83	6.0	3	...	20
Monochloroacetic	6.35	6.3	0	...	0
Citric	6.79	8.25	4	...	52
Crotonic	6.8	6.62	0	...	0
Formic	6.78	6.61	3	...	0
Glutaric	6.85	6.60	10	285	53
Glycollic	6.78	5.8	7	...	24
Lactic	6.75	7.96	10	442	61
Malic	6.85	8.5	10	357	63
Malonic	7.0	6.82	10	325	37
Mandelic	6.7	5.95	1	...	0
Oxalic K salt	6.64	5.38	2	...	12
Oxalic Na salt	6.83	5.84	2	...	18
Tannic	6.8	6.5	0	...	0
Tartaric Na salt	6.94	6.17	9	260	39
Tartaric Na K salt	6.7	5.69	8	...	31
Tartronic	6.82	6.2	8	250	36
Succinic	6.9	8.40	10	425	83
Control	6.71	6.25	9	205	46

a. Value not determined.

Alkalinity of the medium in all cases except for two organic acids (tartaric and tartronic acids causing only minor changes) increased with the increase of nutritive acid concentration up to an optimal concentration at about 0.1 molal. Growth in all cases was related to the nutritive acid concentration. The optimal concentration was found to be about 0.05 molal for acetic, citric, and glutaric; about 0.1 molal for adipic, lactic, malic, succinic, tartaric, and tartronic; and about 0.3 molal for malonic acid. Streptomycin yields evidenced by the assays indicated that the concentration of nutritive acid influenced production. Only lactic acid supported sizable production of streptomycin. Its optimal concentration was 0.1 molal.

The effect of these utilizable acids upon the growth of the test organism in the assay plate is nil. Their effect on the assay of streptomycin is practically nil. These results, as means of six determinations, are summarized by expressing them as a ratio of the size of zones of inhibition from streptomycin with acids to that from streptomycin alone—"Acid/Control Index." The computed "Acid/Control Indices" for the acids are acetic 1.02, adipic 1.04, citric 1.02, glutaric 0.90, lactic 1.01, malic 1.08, malonic 1.06, succinic 1.04, tartaric 1.00, and tartronic 1.05. These indices are based upon a mean 17.75 mm. zone of inhibition for the streptomycin control.

DISCUSSION

The discussion offers some summary interpretations from the overall results on three measured responses of the organism; it presents some

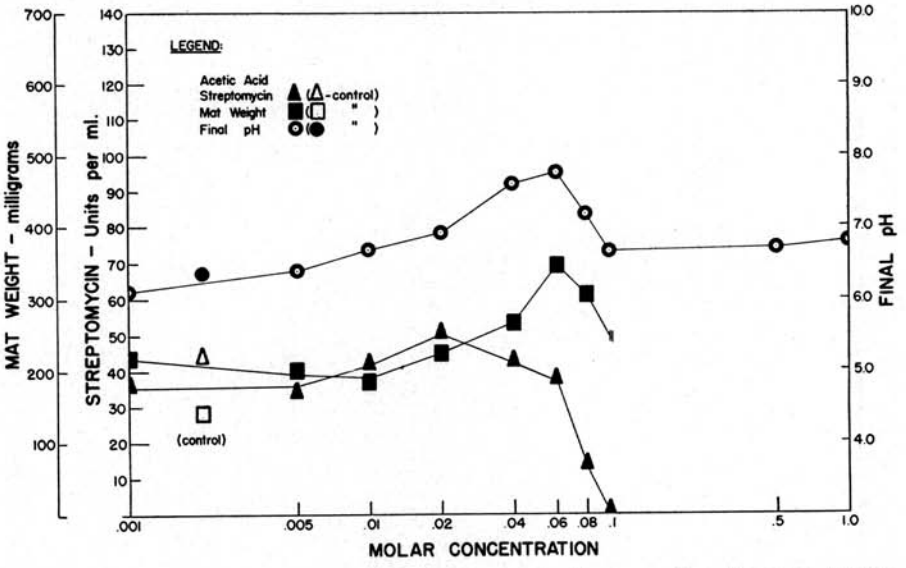

FIG. 1.—Effect of acetic acid at varied concentrations on *Streptomyces griseus* in stationary culture at 26° C.

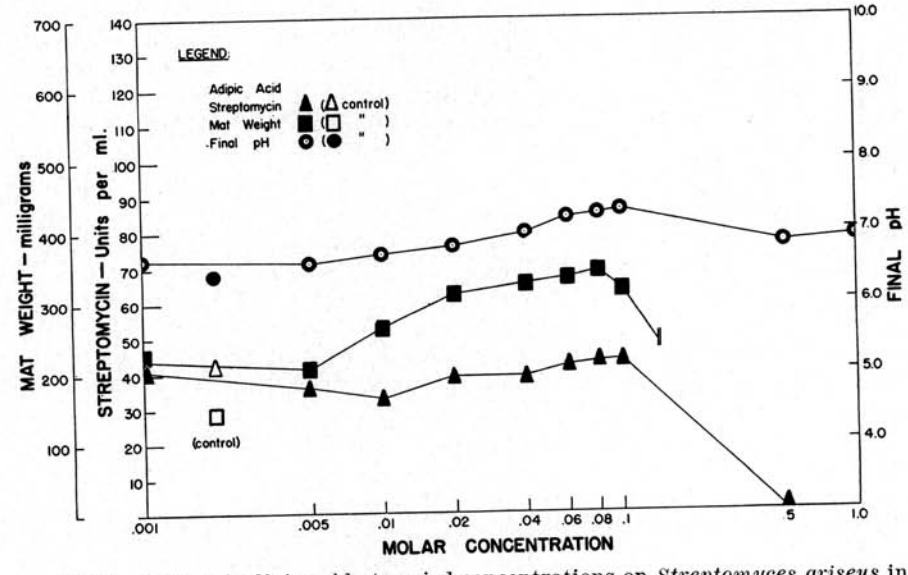

FIG. 2.—Effect of adipic acid at varied concentrations on *Streptomyces griseus* in stationary culture at 26° C.

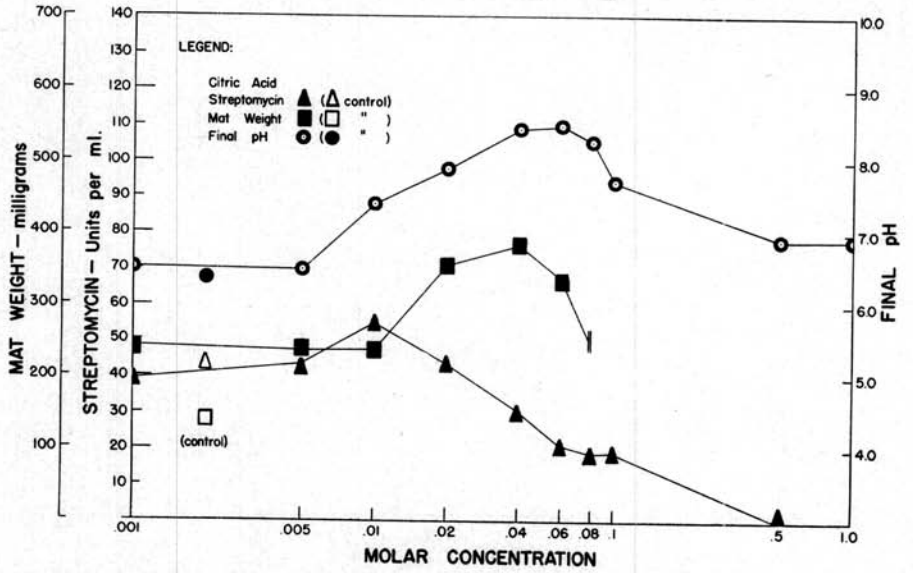

FIG. 3.—Effect of citric acid at varied concentrations on *Streptomyces griseus* in stationary culture at 26° C.

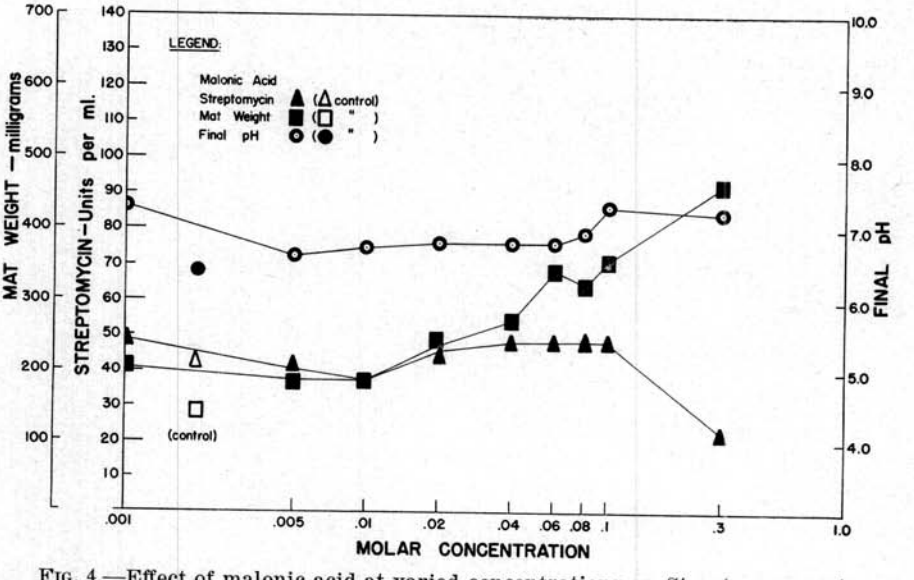

FIG. 4.—Effect of malonic acid at varied concentrations on *Streptomyces griseus* in stationary culture at 26° C.

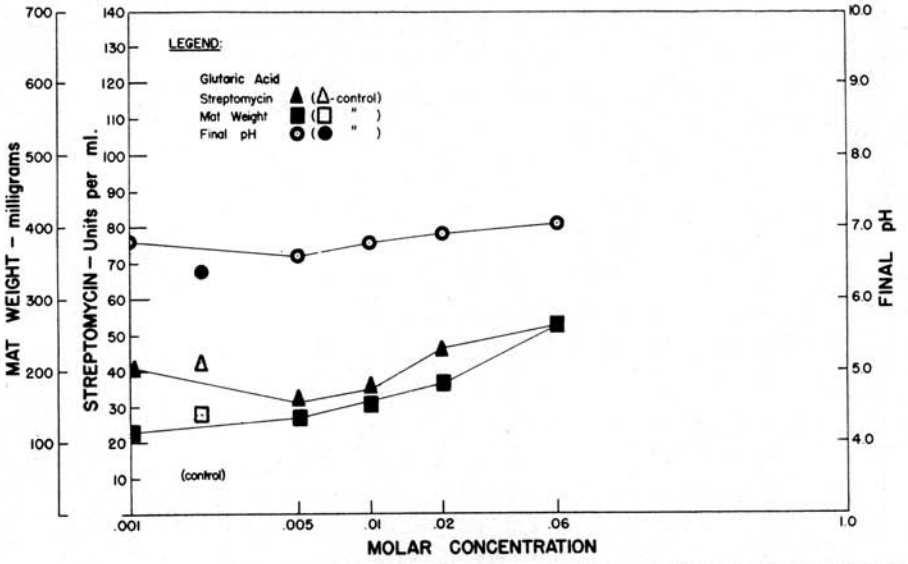

FIG. 5.—Effect of glutaric acid at varied concentrations on *Streptomyces griseus* in stationary culture at 26° C.

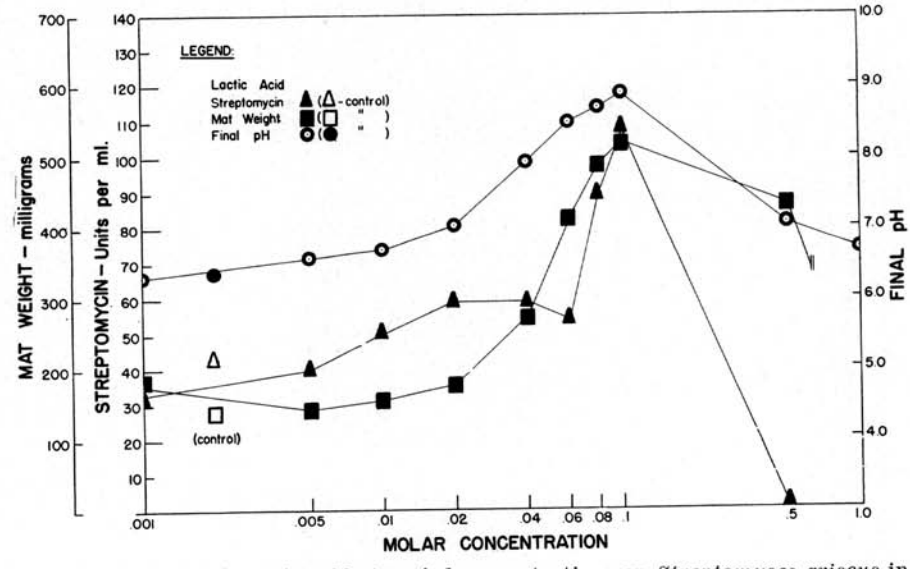

FIG. 6.—Effect of lactic acid at varied concentrations on *Streptomyces griseus* in stationary culture at 26° C.

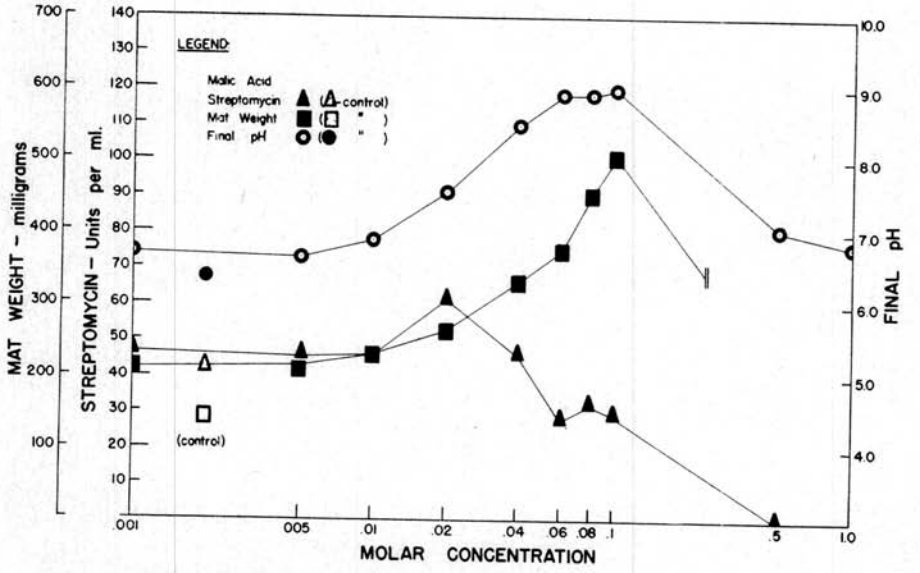

FIG. 7.—Effect of malic acid at varied concentrations on *Streptomyces griseus* in stationary culture at 26° C.

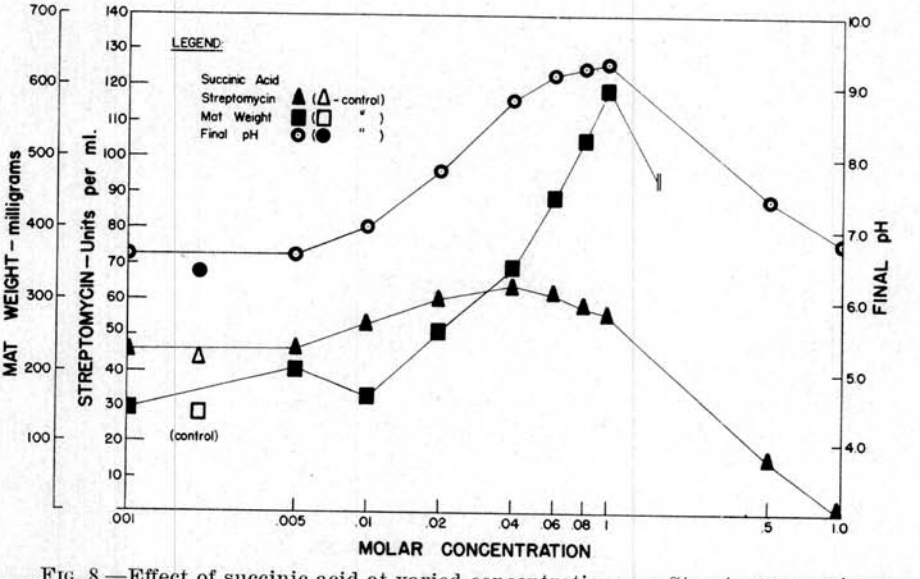

FIG. 8.—Effect of succinic acid at varied concentrations on *Streptomyces griseus* in stationary culture at 26° C.

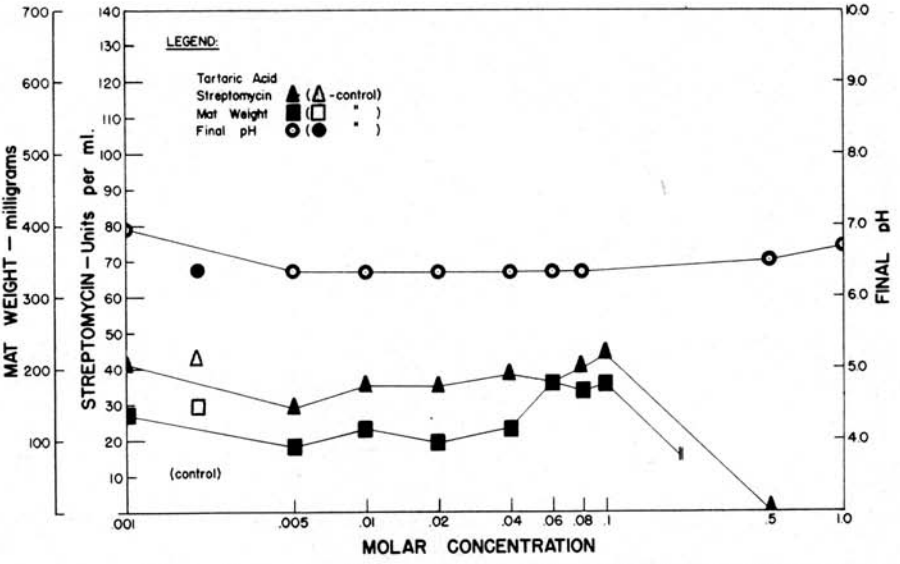

FIG. 9.—Effect of tartaric acid at varied concentrations on *Streptomyces griseus* in stationary culture at 26° C.

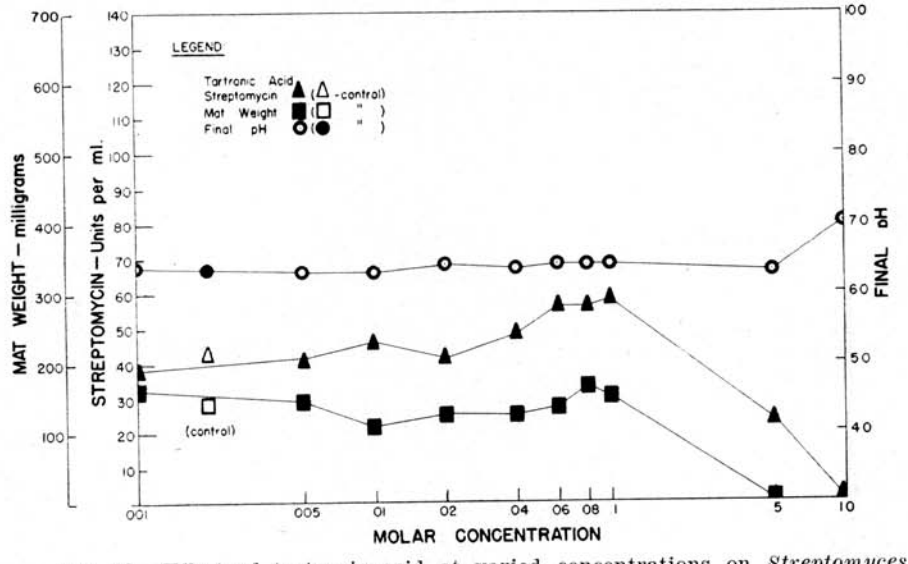

FIG. 10.—Effect of tartronic acid at varied concentrations on *Streptomyces griseus* in stationary culture at 26° C.

considerations with respect to safeguards against artifacts in the data; it advances some speculations relative to the role of trace elements for organic acid utilization, to organic acids influencing metabolic mechanisms, and to lactic acid in streptomycin synthesis. The speculations are included for the sole purpose of attempting to keep the small sector represented by this study oriented at least toward the composite picture of microbial metabolism.

The results from this study extend the information relative to the assimulative and dissimulative capacity of *Streptomyces griseus* for sources of carbon beyond some sugars (17). Although the results are based upon indirect criteria for utilization, they contribute to a preliminary qualitative survey of carbon sources. In addition, the results establish certain basic information by designating some growth inhibitive acids and by indicating differences among utilizable acids.

The three measured responses (pH, growth, and streptomycin production) associated with the metabolism of the organism appear to show a maximum at some range of concentration of each organic acid except for (a) pH in the medium containing glutaric, tartaric, and tartronic acids and (b) streptomycin production in media containing glutaric acid. In the case of glutaric acid, the highest concentration used may have been inadequate to indicate an optimal concentration. An optimal concentration is apparent for each of the other organic acids in the medium as has been found for sugars (17) and for various constituents of the synthetic medium (28, 31). Since these respective optimal concentrations for the three responses occur, for the most part, at different amounts of the acids, they suggest that different metabolic mechanisms are involved in these responses. Similar unparalleled responses are evident in connection with the optimal concentrations of sugars (17) and constituents of the original synthetic medium (28, 31). They have been noted under some other conditions (9, 12). However, the three responses were somewhat parallel with lactic acid, the optimal concentration of which was 0.1 molal for each response.

The H-ion concentration of the medium being related to the balance among ionizable acidic and basic substances at the time of the pH measurements would result from the accumulation of either the residual materials following the assimulation of original constituents of the medium or the end products of metabolism. Metabolites that are consumed as they are produced by the organism would not appreciably affect pH. Thus, the measured increase in alkalinity apparently originates from the accumulation of basic sodium ions or basic end products in the medium. Either of these accumulations would indicate utilization of the acids since the alkalinity is relative to the original concentration of nutritive acid in the medium. It is doubtful whether tartaric and tartronic acids are utilizable (figs. 9, 10). This agrees with another report claiming non-utilization of tartaric acid (23). It is apparent that all the other acids tested at varied concentrations are utilizable (figs. 1 to 8). If organic acids are produced during metabolism, it would appear that these are in turn utilized. It has been reported that organic acids are not produced by Strain No. 4 of *S. griseus* (6) and that the end product of metabolism

of this organism in submerged culture is CO_2 rather than organic acids (8). By escape of this gaseous end product there would be a tendency for any basic non-volatile residues and end products to overbalance the acidic residues and metabolites in the medium. Such could account for the rise in alkalinity usually encountered with this organism. However, if the concentration of glucose is particularly high in a synthetic medium, the reaction does not become more alkaline but becomes acid during fermentation for a period of time (31).

Growth responses of the organism, since they are affected by concentration of the acids, appear to be satisfactory evidence of utilization of these substances. By combining these responses with the somewhat parallel responses in alkalinity changes, these criteria for utilization become more reliable. Growth is better on lactic, malic, and succinic acids than other acids.

Streptomycin production according to the activity detected by the assays is not appreciably increased by any of the acids except lactic acid, a constituent of the original synthetic medium (28,31). Although production from the other acids was not great, there appears to be some relationship of streptomycin yield to concentration of the acids (figs. 1 to 10). Because these optima for streptomycin production with the exception of lactic acid are not the same as those for growth, it appears that the optimal concentration of such nutrients for growth may be "out of balance" for streptomycin production. Along this reasoning, lactic acid at 0.1 molal is "in balance" with other nutrients for growth and streptomycin production simultaneously. As lactic, malic, and succinic acids contributed marked responses in pH and growth, with streptomycin being produced in any appreciable amount only from lactic acid (figs. 6, 7, 8), it appears that lactic acid may specifically contribute to the production of streptomycin by this organism. Mechanisms of metabolism or nutrition favoring the production of lactic acid might conceivably stimulate growth of the organism with appreciable production of streptomycin, whereas good growth without lactic acid being supplied or produced in the medium might result in low yields of streptomycin.

Neither the assay of streptomycin nor the growth of the assay organism on the assay plates was appreciably affected by any of the organic acids at 0.1 molal. However, it has been reported that salts of some of these acids antagonize the action of streptomycin (3, 4, 7, 10, 13, 14, 16) and also effect the growth of test organisms for assay (7, 18, 36). These discrepancies may be explainable in differences among the test organisms used or conditions of the assays. In the paper-disc assay plate method only substances that would diffuse faster than the streptomycin would be able to affect the test organism. In addition, the method is insensitive to substances such as alcohol that might affect the test organisms suspended in a liquid culture (30).

The osmotic concentrations of the various media do not solely influence the optimal concentrations of acids since the optima did not occur at any one concentration. However, the physical effect of the higher concentrations (0.615 molal for 0.5 molal acids) would be expected to influence the functions of the organism (physiological salt solution being 0.155 molal NaCl although based

upon human red blood cells). Since abrupt changes in the responses occurred generally at 0.1 molal (0.215 total molal concentration), the isotonic concentration of the organism (although undetermined) would be expected to be slightly above this total molal concentration. It has been reported that a reduction of NaCl concentration in the medium accounts for a relatively greater amount of streptomycin recoverable from the mycelium than from the medium (9). According to this, the higher concentrations of sodium salts of the organic acids where reduced streptomycin production occurred should favor the release of streptomycin into the medium and thus permit adequate assay of the streptomycin actually formed. Sodium chloride has been reported to stimulate the production of streptomycin in a soybean medium (25).

The relation of trace elements in the medium to the utilization of organic acids is not apparent from the observations and data of this study. However, in other studies trace elements in a medium containing both glucose and lactic acid influenced growth and streptomycin production (28, 31). Also the minerals remaining in the ash of some animal and plant products stimulated growth and streptomycin production (29). In addition to influencing the growth of microorganisms, trace elements are known to alter the metabolism of microorganisms so as to favor the accumulation of commercially desired end products of metabolism such as citric acid (22). Even without the presence of organisms, they can catalyze the oxidation of sugars to organic acids in acid or alkaline solutions when molecular oxygen is supplied (11). They also increase the ionization and reactivity of sugars as acids (11). These phenomena and the fact that some organic acids are utilized by *S. griseus* and other actinomycetes (2, 33) suggest that this organism may utilize organic acids through some mechanism involving metallic trace elements.

Lactic acid appears to play a role in streptomycin production by *S. griseus*. The acid is known to function in CO_2 fixation by *Clostridium butylicum* (5). Carbon dioxide appears to be necessary for some actinomycetes in synthetic media (1) and either CO_2 or the carbonate radical appears to influence streptomycin production by *S. griseus* (31). A lactic acid forming enzyme is inactivated at 37° C. (20), the temperature at which *S. griseus* does not produce streptomycin even though good growth continues (31). Since lactic acid is rapidly consumed by *S. griseus* (31) and is unique among the acids studied for streptomycin production, the synthesis of streptomycin by this organism might conceivably be dependent upon lactic acid which may be supplied to the medium or produced by the organism itself for assimulation or dissimulation (31).

Any effect of streptomycin upon the metabolism of *S. griseus* which produces it is not directly revealed by the data. However, the data do suggest some presumed evidence and inferences for streptomycin effects. Since streptomycin appears to affect the oxidation of acetate by *Escherichia coli* (21) and animal tissue homogenates (32), streptomycin might conceivably influence the oxidative system of *S. griseus* with respect to organic acids if these acids should be broken down to a two-carbon acid such as acetic (15). Such an influence might retard further

synthesis of streptomycin even though growth is excellent when the medium contains nutrients of particular substances such as malic and succinic acids. If lactic acid should afford other or additional mechanisms, the synthesis of streptomycin might not be retarded when lactic acid is available. This would explain the occurrence of higher production of streptomycin from lactic acid than from other acids when growth is approximately equal on the acids concerned.

Another possible explanation is that the acids other than lactic may channel the mechanisms of the organism away from streptomycin synthesis or may direct the mechanisms into streptomycin utilization. Lactic acid, on the other hand, may channel the mechanisms away from streptomycin utilization and toward streptomycin synthesis. Streptomycin is utilized by some microorganisms (24) but no report was found where an organism could produce streptomycin or an antibiotic under some conditions and under certain other conditions could utilize it.

It should be kept in mind that a supposedly pure culture of this organism may mutate into strains (27) or carry strains that possess quite distinct functional characteristics. Such inductions or selections might be brought about by the organic acids in the medium, as growth in these experiments involves successive generations from a small quantity of the inoculum. Organic acids in certain respects might be considered as metabolites which could be more effective than the less oxidized aldehydes (sugars as conventional carbon sources) in influencing metabolic mechanisms. Organic acids are end products of metabolism of many microorganisms (22, 37) and even species of *Streptomyces* (6). The absence of organic acids detectable as end products (6) does not necessarily mean that *S. griseus* is incapable of producing organic acids. It has been reported that *S. griseus* forms very little acid from glucose and no succinic acid during active growth although traces of succionate appear in later stages of growth (6). Organic acids might be formed but if they are rapidly utilized they would not become concentrated sufficiently for detection by usual methods of analysis. The evidence for utilization of organic acids reported herein and mentioned above (6) suggests that organic acids may be significant nutrients or metabolites that influence streptomycin production.

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