# The Production of Neomycin by *Streptomyces Fradiae* in Synthetic Media[1]

HOWARD T. DULMAGE

*Department of Microbiology, New Jersey Agricultural Experiment Station, Rutgers University, New Brunswick, N. J.*

Received for publication November 19, 1952

Of basic importance to the study of antibiotic production by microorganisms is a chemically defined medium in which the effect of various nutrients, both organic and inorganic, can be studied with a minimum of interference from ill-defined substances. Such a medium is reported here for the production of neomycin by *Streptomyces fradiae*, together with studies on the influence of various nitrogen and carbon compounds on this reaction.

## MATERIALS AND METHODS

The *S. fradiae* used in these studies was the original strain isolated by Waksman and Lechevalier (1949) and designated No. 3535 in the N. J. Agricultural Experiment Station Culture Collection. The strain was carried on potato agar (approximate pH 7.0–7.5) in Blake bottles. The bottles were incubated one week at 28 C and stored in the cold until needed.

Growth studies were carried out in shaken culture, 100-ml portions of medium being used per 250-ml Erlenmeyer flask and incubated at 28 C, as in the studies of Swart, Hutchison, and Waksman (1950) on organic media for neomycin production.

Assays were made in triplicate by the cup plate method. The cups were stainless steel 8 mm in diameter. The dilutions were made in 0.1M phosphate buffer (pH 7.0 to 7.1). The test organism was *Bacillus subtilis* ATCC 6633.

All media were autoclaved at 15 pounds pressure for 15 minutes. All carbon sources and $CaCO_3$ were autoclaved separately and added aseptically to the sterile medium. The pH of the media was adjusted with NaOH to 7.0–7.5, before sterilization.

The basic synthetic medium was composed, on a percentage basis, of glucose 0.5, glutamic acid 2.0, $K_2HPO_4$ 0.2, $MgSO_4 \cdot 7H_2O$ 0.05, $FeSO_4 \cdot 7H_2O$ 0.005, $ZnSO_4 \cdot 7H_2O$ 0.005, and $CaCO_3$ 1.0, and was similar to a medium developed by Dulaney (1948) for *Streptomyces griseus*. It was later found that the $CaCO_3$ could be replaced by 0.01 per cent $CaCl_2$. All media were made up to volume with distilled water. Although there were fairly large variations from week to week, the average yield obtained on this medium over a long period was approximately 90 units[2] per ml.

## EXPERIMENTAL

### *Nitrogen Sources*

Table 1 shows the results of an investigation of the suitability of a series of amino acids and a few other organic nitrogen compounds as replacements for glutamic acid in the basic synthetic medium. With the exception of gelatin and 'N–Z Amine A' (Sheffield Farms product), all compounds were added to give a concentration of 0.186 gm nitrogen per liter. The gelatin and the 'N–Z Amine' were added in excess, in a concentration of 50 grams per liter, in the hope that if only a few amino acids could be utilized by this organism, this excess would supply essential amounts of these acids for growth and neomycin production.

The results showed that *S. fradiae* was not able to utilize β-alanine, L-tyrosine, or DL-norvaline for growth; it was able to utilize only very slightly creatine, DL-isoleucine, L-hydroxyproline, L-leucine, DL-methionine, DL-β-phenylalanine, DL-valine, and urea. Moderate growth was obtained when citrulline and L-tryptophane served as nitrogen sources. Growth was good, but delayed, with α-alanine, L-aspartic acid, DL-aspartic acid, L-proline, DL-threonine, and very much delayed with DL-serine. Growth was good, and rapid, with L-arginine, D-glutamic acid, L-glutamic acid, L-histidine, L-lysine, L-proline, and DL-threonine. Best growth was obtained on the complex media containing gelatin or 'N–Z Amine'.

The results indicated that growth of *S. fradiae* can occur without neomycin production. L-arginine and L-lysine were unable to support neomycin production, yet proved excellent nitrogen sources for the growth of this organism. Gelatin, which gave very heavy growth of *S. fradiae*, supported only slight production of neomycin. On the other hand, high yields were not obtained in any case without good growth.

None of the amino acids that yielded neomycin differed greatly in its maximum production. α-Alanine, L-aspartic acid, DL-aspartic acid, D-glutamic acid, L-

[1] Paper of the Journal Series, New Jersey Agricultural Experiment Station, Rutgers University—The State University of New Jersey, Department of Microbiology.

[2] A unit of neomycin is that amount of material which inhibits the growth of a standard strain of *Escherichia coli* in 1 ml of medium.

glutamic acid, L-histidine, L-proline, and DL-threonine, all supported neomycin production in the range of 40 to 85–u/ml, a difference that could not be considered significant in view of the variations obtained from week to week.

TABLE 1. *Effect of organic nitrogen sources on the production of neomycin*

| NITROGEN SOURCE | CONCENTRATION* | NEOMYCIN PRODUCED, UNITS/ML | | |
|---|---|---|---|---|
| | | 3 days | 5 days | 7 days |
| | *gm/l* | | | |
| α-alanine | 11.82 | 22 | 45 | 72 |
| β-alanine | 11.82 | 5 | 3 | 3 |
| L-arginine | 5.79 | 5 | 3 | 3 |
| L-aspartic acid | 17.68 | 5 | 17 | 56 |
| DL-aspartic acid | 35.36 | 5 | 21 | 62 |
| creatine | 5.81 | 5 | 10 | 5 |
| citrulline | 7.76 | 5 | 3 | 5 |
| D-glutamic acid | 19.58 | 21 | 55 | 42 |
| L-glutamic acid | 19.58 | 11 | 41 | 42 |
| L-histidine | 10.37 | 16 | 34 | 32 |
| DL-isoleucine | 34.86 | 5 | 3 | 5 |
| L-hydroxyproline | 17.42 | 5 | 3 | 5 |
| L-leucine | 17.43 | 5 | 3 | 5 |
| L-lysine | 9.71 | 5 | 3 | 7 |
| DL-methionine | 39.66 | 5 | 3 | 5 |
| L-proline | 15.30 | 7 | 54 | 85 |
| DL-β-phenylalanine | 21.93 | 5 | 3 | 5 |
| DL-serine | 13.96 | 5 | 3 | 70 |
| L-tryptophan | 13.57 | 5 | 3 | 5 |
| DL-threonine | 31.66 | 9 | 27 | 85 |
| L-tyrosine | 24.06 | 5 | 3 | 5 |
| DL-valine | 31.13 | 5 | 3 | 5 |
| DL-norvaline | 31.13 | 5 | 3 | 5 |
| urea | 3.99 | 5 | 3 | 5 |
| asparagine | 8.78 | 5 | 11 | 11 |
| gelatin | 50.00 | 29 | 10 | 20 |
| 'N-Z Amine A' | 50.00 | 85 | 146 | 120 |

| *Basal medium* | *gm/l* |
|---|---|
| Glucose | 5.0 |
| $K_2HPO_4$ | 2.0 |
| $MgSO_4 \cdot 7H_2O$ | 0.5 |
| $FeSO_4 \cdot 7H_2O$ | 0.05 |
| $ZnSO_4 \cdot 7H_2O$ | 0.05 |
| $CaCO_3$ | 10.0 |

* Except for gelatin and 'N-Z Amine A,' concentrations adjusted to give 0.186 gm nitrogen per liter.

'N–Z Amine', a casein hydrolysate, supported higher neomycin production than did the individual amino acids, while gelatin yielded only traces of the antibiotic. A comparison of the amino acid composition of casein and gelatin (Hawk, Oser, and Summerson, 1947) reveals that casein has a very high glutamic acid content, while gelatin has a high glycine, proline, and hydroxyproline content. Since glycine and hydroxyproline are unsatisfactory for neomycin production, this may suggest the cause for the marked difference in neomycin production between the two substances. The improved growth of the organism on both of these materials may indicate that for optimum growth a combination of amino acids is required.

The simplest nitrogen sources that can be utilized in a synthetic medium are the inorganic ammonium and nitrate salts. However, these have proved unsatisfactory for neomycin production. Ammonium phosphate and ammonium chloride produced excellent growth of *S. fradiae* in shake flask, but neomycin production was

TABLE 2. *Effect of inorganic nitrogen sources on production of neomycin*

| NITROGEN SOURCE* | GLUCOSE | NEOMYCIN PRODUCED, μ/ML | | |
|---|---|---|---|---|
| | | 3 days | 5 days | 7 days |
| | *gm/l* | | | |
| $(NH_4)_2HPO_4$ | 5.0 | 0 | 0 | 0 |
| $(NH_4)_2HPO_4$ | 10.0 | 0 | 0 | 0 |
| $NH_4Cl$ | 5.0 | 0 | 0 | 0 |
| $NH_4Cl$ | 10.0 | 4 | 8 | 27 |
| $NH_4NO_3$ | 5.0 | 2 | 2 | 2 |
| $NH_4NO_3$ | 10.0 | 9 | 15 | 37 |
| $NaNO_3$ | 5.0 | 4 | 28 | 24 |
| $NaNO_3$ | 10.0 | 8 | 73 | 62 |

| *Basal medium* | *gm/l* |
|---|---|
| $K_2HPO_4$ | 2.0 |
| $MgSO_4 \cdot 7H_2O$ | 0.5 |
| $FeSO_4 \cdot 7H_2O$ | 0.01 |
| $ZnSO_4 \cdot 7H_2O$ | 0.01 |
| $CaCO_3$ | 10.0 |

* Concentration of salts, 5 gm/l.

poor at best. Ammonium phosphate gave rise to no neomycin, whereas ammonium chloride supported only low and delayed production. Ammonium nitrate gave fair growth and low neomycin production. Sodium nitrate produced only poor growth of this organism, but did, on irregular occasions, yield some neomycin in the presence of one per cent glucose. The results of these studies are shown in table 2.

### *Carbon Sources*

Studies on the effect of various carbon compounds on growth and neomycin production of the organism in media containing $NaNO_3$ as a nitrogen source showed that no growth was obtained with sorbose, fructose, sucrose, lactose, mannitol, sodium citrate, sodium lactate, or sodium acetate. Poor growth and low yields were obtained with mannose, maltose, starch, arabinose, sodium fumarate, and sodium malate. Good growth, but no neomycin production, was obtained with glyc-

erol as the carbon source, while sodium succinate gave good growth and average yields of neomycin, although the yields were delayed until late in the growth process. The results are shown in table 3.

The erratic yields obtained with sodium nitrate, as compared to the superior growth and neomycin production with glutamic acid, led to the adoption of the latter as the standard nitrogen source for a synthetic medium.

Glutamic acid, at a concentration of 20 gm per liter, was substituted for glucose in the basal medium, and

TABLE 3. *Effect of various carbon compounds in sodium nitrate media on production of neomycin*

| CARBON SOURCE | CONCENTRATION* | NEOMYCIN PRODUCED, μ/ML | | |
|---|---|---|---|---|
| | | 4 days | 6 days | 8 days |
| | gm/l | | | |
| Glucose | 10.0 | 0 | | 4 |
| Sorbose | 10.0 | | No growth | |
| Fructose | 10.0 | | No growth | |
| Mannose | 10.0 | 18 | 33 | 31 |
| Galactose | 10.0 | 0 | 0 | 0 |
| Sucrose | 10.0 | | No growth | |
| Lactose | 10.0 | | No growth | |
| Maltose | 10.0 | 21 | 29 | 20 |
| Starch | 10.0 | 20 | 23 | 27 |
| Arabinose | 10.0 | 4 | 15 | 20 |
| Mannitol | 10.1 | | No growth | |
| Glycerol | 10.2 | 0 | 0 | 0 |
| Sodium citrate | 16.3 | | No growth | |
| Sodium succinate | 22.5 | 14 | 49 | 56 |
| Sodium fumarate | 9.7 | 0 | 11 | 22 |
| Sodium malate | 11.2 | 0 | 14 | 20 |
| Sodium lactate | 12.5 | | No growth | |
| Sodium acetate | 13.7 | | No growth | |

| *Basal medium* | gm/l |
|---|---|
| $NaNO_3$ | 10.0 |
| $MgSO_4 \cdot 7H_2O$ | 0.5 |
| $FeSO_4 \cdot 7H_2O$ | 0.05 |
| $ZnSO_4 \cdot 7H_2O$ | 0.05 |
| $K_2HPO_4$ | 2.0 |
| $CaCl_2$ | 0.01 |

* Carbon compounds added to give 4 gm C per liter.

several carbohydrates and organic acids were added at a concentration of 5 gm per liter. In this experiment, a control was also run in which no additional carbon source was added, the glutamic acid thus serving as both nitrogen and carbon source. In all cases, final growth was very good, although in absence of any added carbon source, growth seemed to lag somewhat behind that in the other media. The results are shown in table 4.

The control medium gave a maximum yield of 66 u/ml. Sucrose, maltose, and sodium lactate appeared to inhibit slightly neomycin production. Soluble starch, glucose, galactose, and sodium acetate had little effect on the degree of maximum activity, although this maximum was reached 2 days earlier when galactose served as the carbon source. Sodium citrate had a slight stimulatory effect, giving somewhat higher activity than the control. Mannose and arabinose gave significantly higher figures than the control, reaching a maximum of 135 u/ml with the former and 117 u/ml with the latter. Production of activity was delayed until after the fifth day, rising sharply to the final peak on the seventh day. Sodium malate gave the most marked effect of all the carbon sources: by the third day of growth, neomycin production equaled the maximum control figure and continued to climb sharply there-

TABLE 4. *Effect of carbon compounds in glutamate media on production of neomycin*

| CARBON SOURCE* | NEOMYCIN PRODUCED, μ/ML | | |
|---|---|---|---|
| | 3 days | 5 days | 7 days |
| None | 2 | 25 | 66 |
| Glucose | 5 | 42 | 74 |
| Starch (soluble) | 5 | 48 | 75 |
| Maltose | 2 | 25 | 37 |
| Sucrose | 1 | 15 | 24 |
| Mannose | 1 | 8 | 135 |
| Galactose | 12 | 60 | 61 |
| Arabinose | 1 | 15 | 117 |
| Sodium malate | 60 | 145 | 161 |
| Sodium citrate | 14 | 58 | 101 |
| Sodium lactate | 4 | 23 | 26 |
| Sodium acetate | 4 | 41 | 56 |

| *Basal medium* | gm/l |
|---|---|
| Glutamic acid | 20.0 |
| $K_2HPO_4$ | 2.0 |
| $MgSO_4 \cdot 7H_2O$ | 0.5 |
| $ZnSO_4 \cdot 7H_2O$ | 0.05 |
| $FeSO_4 \cdot 7H_2O$ | 0.05 |
| $CaCO_3$ | 10.0 |

* Carbon compounds added, 5 gm/l

after, reaching a peak of 161 u/ml on the final day of growth. The sodium malate employed in this work was a technical grade, and it cannot be definitely stated that the stimulatory effect was due to the malic acid, although there were strong indications that it was.

It is interesting to note that the two organic acids which stimulated neomycin production and the amino acids which supported production (with the possible exception of threonine, about the metabolism of which little is known) are known to be closely associated with the Krebs cycle. Proline and histidine are further removed from this cycle, but have been postulated to form glutamic acid in the immediate process of their metabolism. Until a more detailed study has been made of this relationship, however, one cannot say just what significance this bears to the mechanics of neomycin production.

SUMMARY

Neomycin production could be obtained in synthetic media using sodium nitrate, α-alanine, L-aspartic acid, DL-aspartic acid, D-glutamic acid, L-glutamic acid, L-histidine, L-proline, or DL-threonine as a nitrogen source. Other amino acids and ammonium salts did not support neomycin production. The use of sodium nitrate led to very poorly reproducible results: in one case, sodium succinate when used as the sole carbon source appeared to improve the results obtained with sodium nitrate.

In a glutamate medium, neomycin production could be obtained with no additional carbon source, although the addition of small amounts of glucose improved growth somewhat. In this medium, mannose, arabinose, sodium malate, and sodium citrate gave rise to increased yields when substituted for glucose.

REFERENCES

DULANEY, E. L. 1948 Observations on *Streptomyces griseus*, II. Nitrogen sources for growth and streptomycin production. J. Bact., **56**, 305–313.

HAWK, P. B., OSER, B. L., AND SUMMERSON, W. H. 1947 Practical physiological chemistry. 12th Ed., The Blakiston Co., Philadelphia, Pa.

SWART, E. A., HUTCHINSON, D., AND WAKSMAN, S. A. 1950 Neomycin, recovery and purification. Arch. Biochem., **24**, 92–103.

WAKSMAN, S. A., AND LECHEVALIER, H. A. 1949 Neomycin. a new antibiotic active against streptomycin-resistant bacteria, including tuberculosis organisms. Science, **109**, 305–307.

# Manometric Method for the Evaluation of Microbial Activity of Rumen with Application to Utilization of Cellulose and Hemicelluloses

R. H. McBEE

*Department of Botany and Bacteriology, Montana State College, Bozeman, Montana*

Received for publication November 25, 1952

The ruminant animal and its rumen microorganisms are dependent upon each other for adequate nutrition. Although the relationships between the animal and its rumen population are understood incompletely, it is logical to believe that any major dietary change would be reflected upon the microorganisms long before a change in the nutritional state of the animal could be detected by other means. Thus by selecting the proper procedures it might be possible to measure the potential effects upon the animal of diets deficient in minerals, nitrogenous materials, or other factors before other deficiency symptoms appeared. Similarly, the effect of dietary supplements, antibiotics, urea, etc., might be at least partially determined without prolonged and expensive feeding trials.

The rate of fermentation of various carbohydrates was selected as the criterion of rumen microbial nutrition for this study, because it is easy to measure and because it changes rapidly as the diet is varied. It was found that these fermentation rates could be determined more accurately in the Warburg respirometer than by the method of Quin (1943). The results were quite reproducible if the procedure was carefully standardized and certain precautions observed. This method was used to determine the changes in activity of the rumen microorganisms accompanying the diet variations reported here.

## PROCEDURE

A portion of rumen fluid is removed from a sheep through a permanent fistula using a sampling tube similar to that described by Hungate (1950). The rumen fluid is placed in a loosely stoppered bottle and kept at a temperature of 37 C until it is used, normally a period of less than an hour. Anaerobiosis is maintained by flushing the bottle with carbon dioxide prior to use and by keeping the agitation of its contents to a minimum.

One-ml aliquots of the rumen fluid to be tested are added to Warburg flasks containing equal amounts of 2 per cent sodium bicarbonate buffer in the main part of the vessel and 0.5 ml of the buffered substrate in the side arm. The vessels are flushed with carbon dioxide before and after the addition of rumen fluid. The substrate is dissolved or suspended in a bicarbonate buffer half the concentration of that used to dilute the rumen fluid. The substrate is used in excess, 2 to 5 per cent, in order that its concentration will not be the limiting factor in the fermentation rate.

The vessels are again flushed with carbon dioxide