OBSERVATIONS ON STREPTOMYCES GRISEUS

II. Nitrogen Sources for Growth and Streptomycin Production[1]

EUGENE L. DULANEY

Research Laboratories of Merck & Co., Inc., Rahway, New Jersey

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Synthetic mediums are extremely useful in the investigation of fermentation problems. Because of this fact, various laboratories that are interested in streptomycin have been engaged in developing synthetic mediums for the production of this antibiotic.

VanderBrook *et al.* (1946) reported the use of a near synthetic medium composed of glucose, ammonium sulfate, mineral salts, and 0.1 per cent "curbay B-G." Saunders and Sylvester (1947) used a synthetic medium containing glucose, an organic acid, an inorganic nitrogen compound, and mineral salts. The synthetic medium of Hubbard and Thornberry (1946) was composed of a carbohydrate, the ammonium salt of lactic acid, and mineral salts. It will be noted that these mediums contain inorganic nitrogen compounds or the ammonium salt of an organic acid as the nitrogen source. Although these mediums supported growth and streptomycin production by *Streptomyces griseus*, the reported broth potencies thus obtained were not exceptionally high.

This paper deals with various compounds, both organic and inorganic, as possible nitrogen sources for growth and streptomycin production in a synthetic medium.

MATERIALS AND METHODS

The culture of *Streptomyces griseus* used in these experiments was isolated from a strain originally obtained from Waksman's laboratory. Stock cultures were prepared by adding spore suspensions to tubes of sterile soil. Cultures for inocula were obtained by spreading spores from a soil tube over Blake bottle slants of yeast extract glucose agar. All cultures were derived from the same soil tube. After 7 days' incubation at 28 C, 50 ml of sterile, distilled water were added to each Blake bottle culture and a spore suspension was prepared. One ml of this suspension served as an inoculum for each flask.

The fermentation medium contained the following constituents: glucose 10.0 g, nitrogen source as indicated, NaCl 5.0 g, K_2HPO_4 2.0 g, $MgSO_4 \cdot 7\ H_2O$ 1.0 g, $CaCl_2$ 0.4 g, $FeSO_4 \cdot 7\ H_2O$ 20 mg, $ZnSO_4 \cdot 7\ H_2O$ 10 mg, and distilled water 1 liter. This medium was dispensed in 40-ml amounts in 125-ml Erlenmeyer flasks and sterilized by autoclaving at 121 C for 17 minutes. The pH was adjusted to between 7.0 and 7.5 with N NaOH or N HCl before autoclaving and rechecked after autoclaving. No medium with a pH below 6.5 was used.

[1] Some of these data were presented before the fermentation section of the American Chemical Society in Chicago, April, 1948.

After inoculation, the fermentation flasks were incubated at 28 C on a rotary type shaker moving at 220 rpm so that it described a circle 1 inch in diameter. After growth had started, the fermentation broths were sampled and assayed daily until the maximum streptomycin broth potency had been passed. The agar plate method using a streptomycin calcium chloride complex standard was employed for assay throughout the experiments.

EXPERIMENTAL DATA

Inorganic nitrogen compounds. In order to obtain the simplest medium, the inorganic nitrogen compounds were investigated first. Five compounds, i.e., $NaNO_3$, NH_4NO_3, NH_4Cl, $(NH_4)_2SO_4$, and $(NH_4)_2HPO_4$, were used as single nitrogen sources in the medium noted above at a level of 0.5 per cent. In addition, $CaCO_3$ was added at a level of 0.35 per cent to other replicates except those

TABLE 1

Inorganic nitrogen sources

NITROGEN SOURCE	STREPTOMYCIN BROTH POTENCY—μG PER ML AFTER					
	3 days	4 days	5 days	6 days	7 days	8 days
$NaNO_3$	No growth					
$NaNO_3 + CaCO_3$	No growth					
NH_4NO_3	3	6	4	4	4	3
$NH_4NO_3 + CaCO_3$	13	16	20	45	59	64
NH_4Cl	0	0	0	0	0	0
$NH_4Cl + CaCO_3$	8	14	19	32	22	—
$(NH_4)_2SO_4$	6	4	3	3	2	2
$(NH_4)_2SO_4 + CaCO_3$	7	7	8	23	29	29
$(NH_4)_2HPO_4$	—	142	178	155	155	—

containing $(NH_4)_2HPO_4$. The streptomycin broth potencies obtained in these fermentation mediums are given in table 1; the specific pH changes are omitted.

Streptomyces griseus will not utilize nitrate as a sole source of nitrogen, hence no growth occurred in the medium containing $NaNO_3$ as the nitrogen source. Ammonia, however, is readily utilized. Thus, in the medium containing NH_4NO_3 as the sole nitrogen source, the ammonium nitrogen is utilized with a resultant release of the nitrate. The organism grows until the medium becomes too acid for further growth. Comparable growth and pH changes occur in the mediums containing NH_4Cl and $(NH_4)_2SO_4$ as individual nitrogen sources. The addition of $CaCO_3$ to the fermentations keeps the pH relatively high and allows the organism to grow and produce streptomycin, though at relatively low levels. When $(NH_4)_2HPO_4$ serves as the sole nitrogen source, the pH remains relatively high and yields of approximately 150 μg per ml are consistently obtained. The level of phosphate in this medium is quite important. The use of $(NH_4)_2HPO_4$ at a level of 1.0 per cent, with the fermentation conditions used, will result in little or no streptomycin formation. This phosphate effect is interesting and warrants further investigation. It apparently is not a pH effect. In practice the $(NH_4)_2HPO_4$ is employed at a level of 4.0 g per liter.

Effect of adding complex organic materials to the glucose $(NH_4)_2HPO_4$ *medium.* The addition of various complex organic materials at a level of 0.1 per cent to the glucose $(NH_4)_2HPO_4$ medium has a marked effect on the fermentation. Growth occurs more readily, there is an earlier increase in streptomycin broth potency, and significantly higher streptomycin levels are reached. The effect of these organic materials on the yields of streptomycin can be seen in table 2. The most remarkable increase in broth potency has been obtained by the addition of corn steep solids. The addition of only 0.1 per cent of such solids results in an increase in the streptomycin broth potency of approximately 100 per cent.

The stimulation obtained by the addition of corn steep solids to the glucose $(NH_4)_2HPO_4$ medium cannot be reproduced by the substitution of an equivalent amount of corn steep ash for the corn steep solids. The addition of 0.1 per cent yeast nucleic acid to the glucose $(NH_4)_2HPO_4$ medium also has no stimulating effect. Moreover, the strain of *Streptomyces griseus* used in these experiments exhibits no vitamin deficiency. Small amounts of a number of organic acids, namely, acetic, lactic, fumaric, succinic, pyruvic, malic, and citric also were

TABLE 2

Effect of adding complex organic materials to the glucose $(NH_4)_2HPO_4$ *medium*

MATERIAL ADDED, 0.1 PER CENT	STREPTOMYCIN BROTH POTENCY—μG PER ML AFTER					
	3 days	4 days	4 days	6 days	7 days	8 days
Soybean meal	83	139	200	210	230	215
Corn steep solids	119	228	303	210	—	—
Trypsin digested casein	112	217	224	249	252	—
None	24	135	153	151	—	—

added individually to the medium. In no instance was a stimulation of streptomycin production observed.

The possibility exists that this stimulation is due to organic nitrogen compounds in the corn steep, perhaps a single amino acid or a combination of amino acids. This possibility was investigated by adding 21 amino acids, creatine, urea, and guanidine separately at a level of 0.1 per cent to the glucose $(NH_4)_2HPO_4$ medium. The results are given in table 3.

It may be noted that the medium to which no organic nitrogen compound was added supported streptomycin yields of 153 μg per ml. The marked stimulation due to the addition of corn steep solids and trypsin-digested casein was again obtained. None of the other compounds had a stimulatory effect. Growth was sparse in the flasks supplemented with cysteine and DL-norleucine.

These data indicate that the stimulatory effect of the corn steep solids is not due to a single amino acid that they contain. However, the stimulation may be due to a specific combination of amino acids.

Organic nitrogen compounds. Although individual amino acids had no stimulatory effect when added at low concentrations to the glucose $(NH_4)_2HPO_4$ medium, this does not exclude the possibility that some of these compounds could replace the $(NH_4)_2HPO_4$ in this medium. The utilization of 33 organic nitrogen

compounds as possible nitrogen sources in this synthetic medium was thus investigated by substituting them individually for the $(NH_4)_2HPO_4$. These compounds were used at a level of 0.186 per cent nitrogen. The $(NH_4)_2HPO_4$ was used at a level of 0.085 per cent nitrogen. The streptomycin yields obtained in these fermentations are given in table 4.[2]

TABLE 3

Effect of adding single organic nitrogen compounds to the glucose $(NH_4)_2HPO_4$ medium

COMPOUND ADDED, 0.1 PER CENT	MAXIMUM STREPTOMYCIN BROTH POTENCY	DAY OF MAXIMUM
	μg per ml	
DL-Alpha-alanine	155	5
Glycine	116	7
L-Arginine HCl	135	6
L-Aspartic acid	125	6
L-Cysteine HCl	0	—
L-Cystine	105	5
Creatine hydrate	163	7
L-Glutamic acid	115	5
L-Histidine HCl	145	5
Hydroxy-L-proline	135	6
DL-Isoleucine	127	7
L-Leucine	137	5
DL-Lysine HCl	139	6
DL-Methionine	84	5
DL-Norleucine	0	—
DL-Phenylalanine	166	7
L-Proline	175	5
DL-Serine	155	5
DL-Threonine	110	7
DL-Tryptophan	129	7
L-Tyrosine	176	6
DL-Valine	168	5
Urea	136	6
Guanidine nitrate	133	6
0.1 per cent corn steep solids	300	5
0.1 per cent casein digest	249	6
None	153	5

A yield of 166 μg per ml was obtained in the medium containing $(NH_4)_2HPO_4$. Of the 33 organic nitrogen compounds, 16 supported growth and streptomycin production. However, only 6 of these compounds, i.e., DL-alpha-alanine, beta-alanine, L-histidine HCl, glycine, L-proline, and L-arginine HCl, supported yields in excess of 100 μg per ml.

The use of glycine as the sole nitrogen source resulted in a yield of 151 μg per

[2] In previous experiments Dr. L. E. McDaniel of the Merck Laboratories had tested proline and a number of other amino acids as single nitrogen sources in a synthetic streptomycin fermentation medium.

ml. However, if phenylglycine or *para*-hydroxyphenylglycine is substituted for glycine, no growth occurs.

In the medium containing alpha-alanine (alpha-amino-propionic acid), streptomycin broth potencies of 234 μg per ml were obtained. Beta-alanine

TABLE 4

Organic nitrogen compounds

NITROGEN SOURCE	MAXIMUM STREPTOMYCIN BROTH POTENCY	DAY OF MAXIMUM
	μg per ml	
DL-Alpha-alanine	234	5
Beta-alanine	221	8
DL-Benzoylalanine	Very slight growth	—
Glycine	151	5
N-Phenylglycine	No growth	—
p-Hydroxyphenylglycine	No growth	—
L-Arginine HCl	101	10
L-Aspartic acid	12	5
DL-Alpha-amino-N-butyric acid	Very slight growth	—
DL-Alpha-amino-caprylic acid	Very slight growth	—
L-Cysteine HCl	0	—
L-Cystine	0	—
Creatine hydrate	0	—
L-Glutamic acid	4	4
Hippuric acid	Very slight growth	—
L-Histidine HCl	112	4
Hydroxy-L-proline	1–2	6
DL-Isoleucine	39	5
L-Leucine	5	6
DL-Lysine HCl	0	—
DL-Methionine	0	—
DL-Norleucine	0	—
DL-Phenylalanine	0	—
L-Proline	800	9
Taurine	Growth very poor	—
Sarcosine	No growth	—
DL-Serine	3	8
DL-Threonine	5	6
DL-Tryptophan	0	—
DL-Tyrosine	0	—
DL-Valine	3	8
Urea	3	6
Guanidine nitrate	5	6
$(NH_4)_2HPO_4$	166	5

(beta-aminopropionic acid) supported streptomycin yields of 221 μg per ml, though maximum titers were reached later than when alpha-alanine was used. Any substitution at the third carbon in alanine resulted in lowered yields. For example, L-histidine HCl (beta-imidazole-alpha-aminopropionic acid) supported

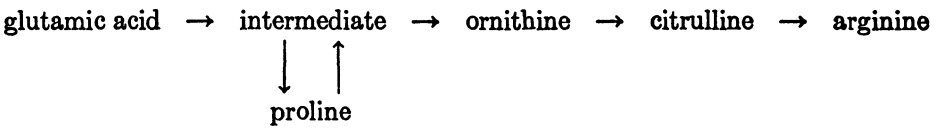
streptomycin yields of only 112 μg per ml. The addition of the imidazole ring resulted in a decrease in potency of approximately 50 per cent. DL-Serine (beta-hydroxy-alpha-aminopropionic acid), DL-valine, (beta-dimethyl-alpha-aminopropionic acid), and DL-isoleucine (beta-methyl-beta-ethyl-alpha-amino-propionic acid) supported even lower streptomycin broth titers.

When L-arginine HCl was used as the sole nitrogen source, growth was slow and maximum yields of streptomycin were reached late. It should be noted that arginine is the only amino acid containing more than three carbons in a straight chain that supports relatively high yields of streptomycin.

Striking results were obtained by the use of L-proline as the sole nitrogen source. There was an increase in mycelium weight, and streptomycin broth potencies of 800 μg per ml were obtained. There was, however, a lag in both growth and streptomycin production.

Attempts to substitute structurally related compounds for the proline have met with failure. The use of hydroxyproline as the sole nitrogen source results in poor growth and little if any streptomycin production. Pyrrolidone-carboxylic acid and N-acetyl proline, when substituted for the L-proline, do not support growth. The same is true for pyrrole.

It has been shown that the ornithine cycle proposed by Krebs and Henseleit (1932) exists in *Neurospora crassa* (Srb and Horowitz, 1944). Moreover, the metabolism of proline in the rat (Shemin and Rittenberg, 1945; Stettin and Schoenheimer, 1944) is closely linked to this ornithine cycle. In addition, Bonner (1946) indicates that in *Penicillium* ornithine and proline have a common precursor, with glutamic acid the precursor of this proposed intermediate. He represents the synthesis of proline and arginine in *Penicillium* by the following scheme:



It would appear that this scheme does not hold true for *Streptomyces griseus*. Glutamic acid, when substituted for proline, supports good growth but variable and usually low streptomycin yields. The use of arginine as the sole nitrogen source results in fair growth and streptomycin broth potencies of approximately 100 μg per ml. None of the other valeric derivatives has even supported growth in mediums to which it was added as an individual nitrogen source. Alpha-aminovaleric acid, delta-aminovaleric acid, and alpha-delta-diaminovaleric acid (ornithine) were the compounds tested. In addition, N-valeric acid when used as the sole carbon source in a medium containing $(NH_4)_2HPO_4$ as the source of nitrogen did not support growth.

The high broth potencies that have been obtained in the proline medium raise the possibility of adding proline to other mediums and thus obtaining increased streptomycin yields in these mediums. In preliminary experiments, the addition of proline at a level of 0.25 per cent to a medium containing $(NH_4)_2HPO_4$, casein

digest, or soybean meal as the nitrogen source resulted in no increase in streptomycin broth titers. The results reported in a previous section showed that single amino acids, when added at a level of 0.1 per cent to the glucose $(NH_4)_2HPO_4$ medium, did not increase streptomycin broth potencies. In addition, L-proline, glycine, DL-alpha-alanine, L-arginine HCl and L-histidine HCl were added singly to the glucose $(NH_4)_2HPO_4$ medium at a level of 0.5 per cent. All possible combinations of these compounds also were added to the same medium in such quantities that the total amount of added amino acids was always 0.5 per cent. Casein digest and proline-rich gelatin also were tested. The yields of streptomycin obtained in these fermentations are shown in table 5.

TABLE 5

The effect of adding amino acids singly and in combination to the glucose $(NH_4)_2HPO_4$ medium

MATERIAL	TOTAL AMOUNT	MAXIMUM STREPTOMYCIN BROTH POTENCY	DAY OF MAXIMUM
	per cent	*μg per ml*	
DL-Alpha-alanine	0.5	56	5
L-Arginine HCl	0.5	61	6
Glycine	0.5	68	6
L-Proline	0.5	170	6
L-Histidine HCl	0.5	56	6
Proline, alanine, glycine, histidine, arginine	0.5	51	6
Proline, alanine, glycine, histidine	0.5	55	5
Proline, alanine, glycine	0.5	51	6
Proline, alanine	0.5	59	5
Alanine, glycine, histidine, arginine	0.5	41	5
Alanine, glycine, histidine	0.5	51	5
Alanine, glycine	0.5	30	5
Glycine, histidine, arginine	0.5	31	5
Glycine, histidine	0.5	66	6
Histidine, arginine	0.5	50	5
Casein digest	0.5	275	5
Casein digest	0.3	274	5
Gelatin	0.5	195	5
None	—	144	6

With the exception of L-proline at 0.5 per cent the addition of the amino acids, singly and in combination, resulted in decreased streptomycin yields. The increased potencies due to the addition of 0.5 per cent L-proline is significant but not striking. Gelatin at 0.5 per cent also increased yields significantly as did casein digest at 0.3 per cent and 0.5 per cent. The increase due to the use of casein digest is marked. The addition of compounds that are metabolically related to proline, e.g., ornithine, has not resulted in a stimulation of streptomycin production.

Although no specific data are given, it also should be noted that, when other nitrogenous compounds are added to the synthetic medium containing L-proline as the principal source of nitrogen, the streptomycin yields thus obtained are

much lower than are obtained with proline alone. This is not true if the level of added nitrogenous compounds is very low.

These data certainly indicate that L-proline when used as the sole nitrogen source is capable of supporting high streptomycin broth potencies. Although it is true that increased mycelium weights are obtained in this L-proline medium, excellent growth can be obtained in mediums that support little or no streptomycin formation.

The data presented thus far raise a number of interesting and important questions, the answers to which will have to await the results of metabolism studies now in progress. Of particular importance will be the mechanism of proline utilization in different mediums, i.e., a medium in which proline is the sole nitrogen source and a medium in which it is used in conjunction with other nitrogen compounds, such as $(NH_4)_2HPO_4$.

ACKNOWLEDGMENT

The mineral salts used in the basal medium are essentially those used by Dr. L. E. McDaniel in his unpublished research on synthetic streptomycin fermentation mediums.

Some of these data have been discussed with Drs. E. E. Howe and R. L. Peck. The helpful suggestions and criticisms of Dr. David Perlman are gratefully acknowledged. The N-acetyl proline was prepared by Dr. Peck. Dr. Perlman prepared the pyrrolidone-carboxylic acid.

SUMMARY

A number of organic and inorganic nitrogen compounds have been tested as possible nitrogen sources in a synthetic streptomycin fermentation medium.

Nitrate nitrogen is not utilized by the streptomycin-producing strains of *Streptomyces griseus* that have been studied, but ammonium nitrogen is readily available.

A simple synthetic medium containing ammonium nitrogen has been devised. This medium contains glucose 10.0 g, $(NH_4)_2HPO_4$ 4.0 g, NaCl 5.0 g, K_2HPO_4 2.0 g, $MgSO_4 \cdot 7H_2O$ 1.0 g, $CaCl_2$ 0.4 g, $FeSO_4 \cdot 7H_2O$ 20.0 mg, $ZnSO_4 \cdot 7H_2O$ 10.0 mg, and distilled water 1 liter. Streptomycin broth potencies of 150 μg per ml are consistently obtained in this medium. The level of phosphate is important.

The addition of small amounts of corn steep solids, casein digest, and soybean meal to this medium results in increased titers of streptomycin. If amino acids are added singly to this medium, there is no stimulation of streptomycin production.

Thirty-three organic nitrogen compounds were tested as possible substitutes for the $(NH_4)_2HPO_4$ in the synthetic medium. Six of these compounds, namely, DL-alpha-alanine, beta-alanine, L-histidine HCl, L-arginine HCl, glycine, and L-proline, supported yields of streptomycin in excess of 100 μg per ml. Yields of 800 μg per ml were obtained by the use of L-proline as the sole nitrogen source.

The use of L-proline as a supplement did not increase streptomycin broth potencies.

A number of compounds that are structurally related to L-proline have been tested as substitutes for the proline. None of them could replace this amino acid.

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