STUDIES ON THE PHYSIOLOGY OF A STREPTOMYCIN-PRODUCING STRAIN OF STREPTOMYCES GRISEUS ON PROLINE MEDIUM

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Complex organic mediums have generally been considered necessary for the production of high yields of streptomycin by *Streptomyces griseus* (Waksman, Schatz, and Reilly, 1946). Quantities of streptomycin approaching 1 gram per liter have been reported from the growth of mutant strains on such mediums (Stanley, 1947). This represents a conversion of 5 to 10 per cent of the organic constituents of the medium to streptomycin. Few attempts have been made to define conditions regulating the conversion or to study the mechanism of streptomycin formation.

Dulaney and Perlman (1947) have reported observations of the biochemical activities of *S. griseus* under conditions favorable for streptomycin production. Two stages of activity in a glucose peptone meat extract medium were described. During the growth phase, the production of mycelium was accompanied by a reduction in the soluble constituents of the medium (N, C, P), fermentation of the available carbohydrate, a high oxygen demand, and little production of streptomycin. During the autolytic phase, the mycelium weight decreased markedly, inorganic phosphorus and soluble nitrogen were released into the medium, the oxygen demand dropped, and considerable quantities of streptomycin were produced.

In the preceding paper from this laboratory (Dulaney, 1948), a synthetic medium containing proline is described that supports streptomycin production equal to or better than that obtained with organic mediums. The stages of the biochemical activities of *S. griseus* on this medium are outlined in figure 1. The first stage, that of cell growth not associated with streptomycin production, is prolonged in the proline medium, primarily because of an extended lag phase before proline is attacked or growth occurs. In phase 2 the remaining amounts of glucose and proline are utilized, accompanied by rapid accumulation of streptomycin. Finally, lysis of the culture occurs.

The specific action of proline, among amino acids, in allowing high streptomycin yields, offers the opportunity for study of the mechanism of streptomycin formation. As a part of this investigation, methods were sought to decrease the lag phase of growth. These included an attempt to promote growth by the utilization of adapted *S. griseus* inoculum, the addition of essential nutrients, and the study of products intermediate in proline decomposition that were more susceptible than proline to attack by *S. griseus*, but were still conducive to high streptomycin yields.

Analytical methods. With the exception of the determination of proline, standard analytical procedures were used. Glucose was determined as a re-

ducing sugar by the method of Somogyi, total nitrogen by micro-Kjeldahl procedure, NH_4-N by steam distillation from 5 per cent Na_2CO_3 solution, and total carbon by the wet combustion method of McCready and Hassid (1942). Streptomycin was determined by the FDA cup assay. Proline determinations were made by an unpublished procedure, developed in our laboratories and based on the amino acid assay procedures described by Stokes, Gunness, Dwyer, and Caswell (1945). *Leuconostoc mesenteroides* was employed as the assay organism.

Most of the fermentations were conducted in 250-ml Erlenmeyer flasks containing 25 ml of medium and were incubated at 28 C on a rotary shaker operating

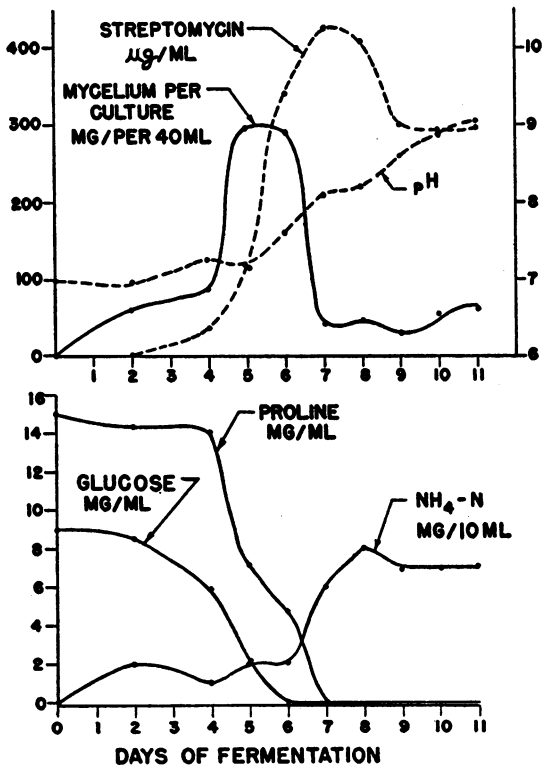

Figure 1. Fermentation characteristics of *S. griseus*.

at 220 rpm. The *S. griseus* inoculum was derived from strains previously found satisfactory for streptomycin production (Schatz and Waksman, 1945).

EXPERIMENTAL PROCEDURE AND RESULTS

Development of "adapted" cells. *S. griseus* was grown from a spore inoculum on a synthetic basal medium containing 0.57 per cent $(NH_4)_2HPO_4$ or on 1 per cent proline, which contains an equivalent amount of nitrogen (Dulaney, 1948). Mycelial growth was considered suitable for transfer after attaining about 80 per cent of its maximum weight.

An attempt to show the adaptability of the organism to proline was made by

10 per cent transfer of vegetative inoculum to fresh culture medium (table 1). There was no evidence that cells became adapted to use proline. Cells developed on proline had a lag phase equal to that of those developed on $(NH_4)_2HPO_4$ medium, when inoculated into proline fermentation medium. Equivalent results were obtained when 100 per cent transfer was made of washed cells. The lag was much reduced, but equal in the case of inoculum developed on either $(NH_4)_2HPO_4$ or proline medium.

A striking difference was noticed in streptomycin yields from fermentations initiated with the two types of vegetative inocula. Only those cultures receiving the inoculum developed on proline medium produced high streptomycin yield. This suggests that the specific action of proline in increasing streptomycin production is not due to a direct precursor action but results from a more general effect on the physiological activity of the cellular substrate.

TABLE 1

The effect of the medium used for inoculum development on streptomycin production and proline utilization

INOCULUM DEVELOPMENT			FERMENTATION IN PROLINE MEDIUM	
Nitrogen constituent	Maximum streptomycin produced	Inoculum size	Streptomycin produced	Day of proline utilization
	μg/ml	*per cent*	*μg/ml*	
$(NH_4)_2HPO_4$*	175	10	200	3
Proline†	960	10	930	3½
$(NH_4)_2HPO_4$	115	100	375	2½
Proline	930	100	525	2½

* Used as vegetative inoculum on fourth day.

† Used as vegetative inoculum on seventh day.

Requirement for NH_4-N. The synthetic basal medium, containing a mixed N source composed of $(NH_4)_2HPO_4$ and proline, showed no lag in proline utilization, despite the fact that there was ample nitrogen present as NH_4-N for growth of the culture. Complete proline decomposition, NH_4-N utilization, and maximum growth coincided on the fourth day of incubation from a spore inoculum, as compared with seven days on proline alone. *S. griseus* has great difficulty in utilizing proline for the supply of its total nitrogen demands. However, if a trace quantity of NH_4-N is present to supply an essential requirement for unknown synthetic processes, proline can adequately supply the major portion of the nitrogen needs of the microorganism. A correct balance of NH_4-N can be found that will speed the rate of growth of *S. griseus* to a maximum but will not interfere with the development of mycelium of high streptomycin-producing capacity. An optimum of 0.02 per cent $(NH_4)_2HPO_4$ with 1 per cent proline was found (table 2).

Carbon-nitrogen balance. Proline utilization, once initiated, is very rapid. NH_4-N does not accumulate during the decomposition. It is evident that intermediate nitrogenous compounds must accumulate in the medium. During the

utilization of these intermediates streptomycin production occurs. Graphs representing the carbon and nitrogen balance at various incubation periods in the proline fermentation are shown in figures 2 and 3. The medium was the proline medium described in the preceding paper (Dulaney, 1948), which contained 1.5

TABLE 2

Streptomycin production on a proline medium with the amount of $(NH_4)_2HPO_4$ as a variable

$(NH_4)_2HPO_4$ CONCENTRATION	STREPTOMYCIN YIELD ON 8TH DAY
per cent	*μg/ml*
0.57	225
0.20	170
0.06	570
0.02	675
0.006	530
0.002	390
0.0006	200
0.0002	130
—*	25

$(NH_4)_2HPO_4$ medium control, maximum on seventh day, 120 μg.

* Maximum on proline medium was reached on eleventh day of incubation, 430 μg.

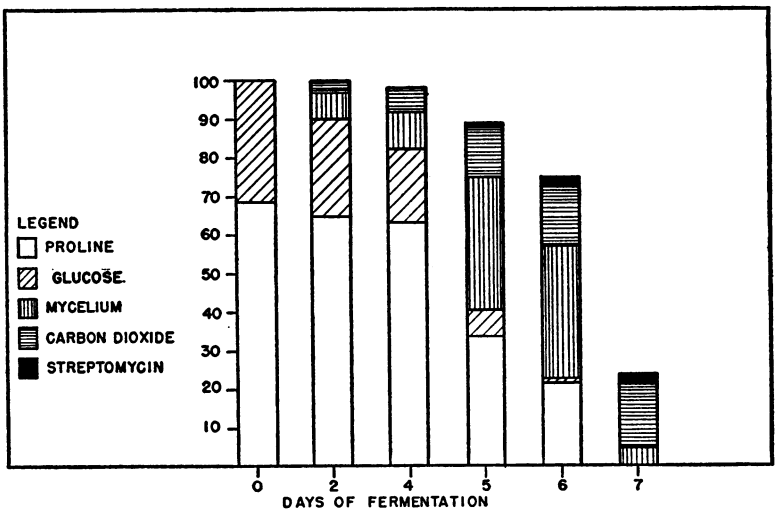

Figure 2. Carbon balance of *S. griseus* fermentation.

per cent L-proline and 1 per cent glucose. During the maximum rate of mycelium accumulation, between the fourth and sixth day, unaccounted-for nitrogen was present. An associated quantity of unaccounted-for carbon also points to the accumulation of intermediates. After lysis, on the seventh day, a large portion of unmeasured soluble nitrogen compounds was liberated into the medium.

Proline is not utilized by *S. griseus* by a direct rapid deamination reaction,

since NH_4-N does not accumulate. Reductive ring rupture to delta-aminovaleric acid, shown to occur with *Clostridium sporogenes* by Strickland (1945), is not the major reaction with *S. griseus*. Neither this compound nor alpha-aminovaleric acid as the N source will support growth of *S. griseus*, either alone or combined with 0.02 per cent $(NH_4)_2HPO_4$. The oxidative product, alpha-keto-delta-aminovaleric acid, shown by Blanchard, Green, Nocito, and Ratner (1944) to be the product of proline breakdown by an enzyme isolated from rat kidneys and livers, was not available for test as a supplement to the culture medium. However, a very small quantity of an ether-insoluble 2,4-dinitrophenylhydrazone was crystallized from fermentation broths corresponding to the 5th- and 6th-day cultures of figures 2 and 3. No crystals were obtained from an old lysed culture. The crystals, after washing with water and drying with ether, melted at 210 to 220 C. They resemble the 2,4-dinitrophenylhydrazone of alpha-keto-delta-aminovaleric acid (Krebs, 1939). Reversal of the scheme proposed for proline synthesis by *Penicillium notatum* (Bonner, 1946) does not seem probable for proline utilization by *S. griseus*.

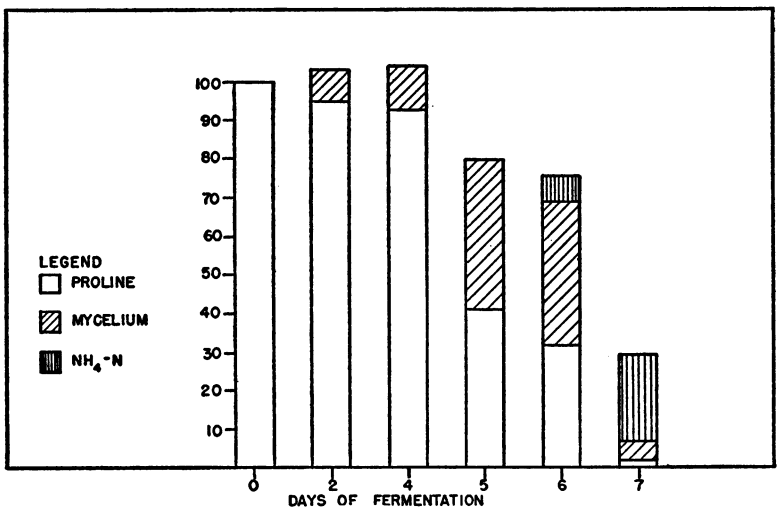

Figure 3. Nitrogen balance of *S. griseus* fermentation.

glutamic acid → intermediate → ornithine → citrulline → arginine

↑ ↓

proline

Ornithine is not used for growth. Glutamic acid, while utilized, supports only low yields of streptomycin.

Resting cell suspensions have been used to prepare the intermediate(s) in quantity. Viable *S. griseus* cells, washed well with phosphate buffer and concentrated in volume 5 times, decomposed 0.25 mg proline per ml per hour with little NH_4-N formation.

The intermediate product produced by this method led to high yields of streptomycin by *S. griseus* when it was sterilized by filtration and added to a synthetic medium as the source of nitrogen. The intermediate is an amino-containing compound. Practically all the soluble nitrogen present at the time of maximum mycelial development may be accounted for as residual proline, NH_4-N, or amino nitrogen. The amino compound was not glutamic acid, aspartic acid, or any one of the 10 additional amino acids which may be measured by microbiological procedures (Stokes, Gunness, Dwyer, and Caswell, 1945).

DISCUSSION

Proline has a specific effect in increasing streptomycin production. Although it is interesting to speculate on proline fragments that may be combined into the streptomycin molecule as a precursor, in the manner in which the phenylacetyl radical is combined into benzylpenicillin (editorial in *Science*, 1947), the results of this investigation do not support this mechanism. Proline is utilized in the presence of NH_4-N, but streptomycin production is high only in a minimal concentration of NH_4-N or in its absence. Also, cells grown in the presence of proline are superior as an inoculum for streptomycin production to cells grown in $(NH_4)_2HPO_4$ medium. One cannot ascribe this to a precursor effect, since such cells are usually used in only 10 per cent concentration and they increase as much as ninefold on transfer to a new medium. Streptomycin accumulation, which reaches maximum rate only near the end of the cellular multiplication, is influenced greatly by the inoculum growth medium.

When proline serves as the sole source of nitrogen in a synthetic medium, there is a long lag phase of growth. This study has shown, however, that the lag phase may be reduced appreciably by supplying the culture with trace quantities of $(NH_4)_2HPO_4$. Further studies of the proline decomposition product, which accumulates before NH_4-N or streptomycin is produced, may demonstrate a less expensive nitrogen source that retains the ability to stimulate streptomycin production.

SUMMARY

Yields of 1 gram of streptomycin per liter were produced by *Streptomyces griseus* in a medium containing proline as the sole source of nitrogen. The extended fermentation time on this medium may be reduced by the addition of traces of $(NH_4)_2HPO_4$. Excess NH_4-N causes reduced streptomycin yield.

A vegetative inoculum of *S. griseus* developed on a proline medium is more satisfactory for streptomycin production than is an inoculum produced on $(NH_4)_2HPO_4$ medium. During growth on proline medium, or by the action of washed cells, *S. griseus* produces an unidentified amino-nitrogen-containing intermediate by a mechanism different from that described for other microorganisms. The intermediate acts like proline in supporting increased streptomycin production by *S. griseus*.

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