STREPTOMYCIN FORMATION BY INTACT MYCELIUM OF *STREPTOMYCES GRISEUS*

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ABSTRACT

NOMI, RYOSAKU (Rutgers, The State University, New Brunswick, N.J.). Streptomycin formation by intact mycelium of *Streptomyces griseus*. J. Bacteriol. **86**:1220–1230. 1963.—A study was made of streptomycin formation by intact mycelium of *Streptomyces griseus* 107 grown in glucose-yeast extract medium. When mycelium harvested after 24, 48, and 72 hr was compared, the earliest growth showed the highest activity in producing streptomycin from glucose. The concentration of streptomycin in the mycelium was higher in the older growth. Calcium chloride had a remarkable effect in increasing streptomycin production from the precursors in the mycelium, especially when the mycelium was grown for 48 hr or longer. The effect of calcium chloride cannot be attributed to the precipitation of an excess of inorganic phosphate in the medium. Glucose, fructose, glycerol, lactic acid, glucosamine, streptidine, and inositol stimulated streptomycin formation, whereas gluconic acid, glucuronic acid, streptamine, and strepturea did not. When 24-hr-old mycelium was suspended and shaken in 0.5% glucose solution, the antibiotic precursors necessary to produce streptomycin were found mainly in the supernatant of the culture rather than in the mycelium. The supernatant included some substance which had a weak antibiotic activity. This substance was less basic than streptomycin and was transformed to streptomycin with a remarkable increase in antibiotic activity.

Numerous investigations have been published dealing with the formation of streptomycin by various strains of *Streptomyces griseus* in growing culture, the conditions of its formation, and the effect of culture media, organic and inorganic compounds, and special supplements. The mycelium of the organism was assumed to change its physiological characteristics as well as its morphology through the various stages of the fermentation process; however, these changes resulting from streptomycin formation by the mycelium have not been given much consideration. Such information would be useful in any approach to the relationship between the physiology of the organism and streptomycin production. It may also throw light upon the biosynthetic reactions involved in streptomycin formation. The use of washed mycelium is convenient for this purpose, since any interference by the growth process of the organism can be eliminated under suitable conditions. Studies on the formation of streptomycin by washed mycelium were carried out by Severin and Gorskaya (1959), Schaiberger (1959), and Majumdar and Kutzner (1962). It is clear from their results that streptomycin is produced by washed mycelium from certain constituents in the mycelium and those added to the medium without growth occurring.

Because of the advantages of using washed mycelium, this method was used in an investigation of the physiological characteristics of the mycelium during streptomycin biosynthesis, such characteristics depending on the stage of growth of the organism and the condition of the mycelium. The washed mycelium was able to elaborate streptomycin from various substances added to the medium of the mycelium-suspended culture. A slightly alkaline pH was necessary for streptomycin formation from glucose by the washed mycelium.

MATERIALS AND METHODS

Strain and inoculum. S. griseus 107 (Nomi, 1963) derived from *S. griseus* Z 38 was used in all experiments. One loopful of aerial mycelium from a slant culture on glucose-yeast extract agar was inoculated in 10 ml of the same medium without agar in a 50-ml Erlenmeyer flask and

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cultured on a reciprocating shaker (98 rev/min) at 28 C for 2 days. A 0.5-ml portion of this broth was transferred to 40 ml of the same medium as the above in a 250-ml Erlenmeyer flask and cultured as indicated. The latter broth was used as inoculum for preparation of washed mycelium. The glucose-yeast extract medium consisted of 1% glucose, 1% yeast extract, 0.5% sodium chloride, 0.025% magnesium sulfate, and 0.001% ferrous sulfate (LePage and Campbell, 1946).

Preparation of washed mycelium. A 0.5-ml portion of the above-mentioned inoculum was transferred to 40 ml of glucose-yeast extract medium in a 250-ml Erlenmeyer flask and was cultured as indicated for the necessary period. This fermentation broth was centrifuged, and the mycelium was washed by centrifugation twice with 18 ml of 0.5% sterile sodium chloride solution. After washing, the centrifuged mycelium was made up to 9 or 10 ml with 0.5% sodium chloride solution. The washed mycelium was used immediately after preparation. Dry weight of washed mycelium was determined in each preparation as follows: 3 ml of washed mycelium in an aluminum dish were dried in an oven at 85 to 90 C for 4 to 6 hr, kept in a vacuum desiccator containing calcium chloride for 24 hr, and then weighed. In the determination of mycelium weight in Table 1, the mycelium was centrifuged from 10 ml of fermentation broth. It was then washed once with 10 ml of distilled water, diluted to 10 ml with distilled water, and 8 ml were dried and weighed.

Three methods for washing the mycelium were studied in preliminary experiments. Distilled water left a considerable quantity of streptomycin in the mycelium. Though potassium phosphate buffer solution (pH 7.0, 0.1 M) was most efficient in eluting the streptomycin present in the mycelium, it was felt that the phosphate ion adsorbed onto mycelium during the washing process might have some effect on utilization of carbohydrate in the subsequent experimental process. Therefore, 0.5% sodium chloride solution was adopted as the washing solution, since this salt showed an intermediate eluting efficiency for streptomycin in the mycelium, and also because the glucose-yeast extract medium included 0.5% sodium chloride.

Suspension culture of washed mycelium. Unless otherwise stated, the mycelium-suspended culture consisted of 3 ml of washed mycelium and 14 ml of 0.5% sodium chloride solution which also included the supplements being tested in a 50-ml Erlenmeyer flask. Incubation was carried out under the same conditions as previously mentioned. Dry weight of mycelium per milliliter of each mycelium-suspended culture was calculated from the dry weight of washed mycelium, which was determined by the above-mentioned method.

Sterilization. All sterilization consisted of autoclaving at 121 C for 15 min, except for the sterilization of glucosamine, galacturonic acid, and glucosaminic acid which was done by Seitz filtration. When necessary, neutralization of medium and supplemented compounds was carried out before sterilization.

Determination of pH, streptomycin, and reducing sugar. A glass electrode pH meter was used for pH measurement. Streptomycin was assayed by an agar diffusion method and expressed as micrograms per milliliter of broth or as micrograms per milligram (dry weight) of mycelium in 1 ml of broth. Reducing sugar was determined by the method of Somogyi (1954).

RESULTS

When *S. griseus* 107 was grown in glucose-yeast extract medium, the fermentation process was generally divided into three phases, as indicated in Table 1. The first phase (0 to 24 hr) was characterized by an increase in the pH value

TABLE 1. *Changes during fermentation of a glucose-yeast extract medium*

Time	Glucose-yeast extract medium				Glucose-yeast extract medium plus 0.02% $CaCl_2 \cdot 2H_2O$			
	pH	Strepto-mycin	Residual glucose	Dry wt of mycelium	pH	Strepto-mycin	Residual glucose	Dry wt of mycelium
hr		*μg/ml*	*mg/ml*	*mg/ml*		*μg/ml*	*mg/ml*	*mg/ml*
0	6.75	1.1	10.8	—	6.75	1.1	10.8	—
24	7.8	35	7.8	5.5	7.8	41	7.7	5.4
48	7.6	224	0.4	7.5	7.25	345	0.4	7.4
72	8.05	228	0.4	3.0	7.8	410	0.3	6.2
96	8.4	210	0.4	2.3	8.3	452	0.3	4.9

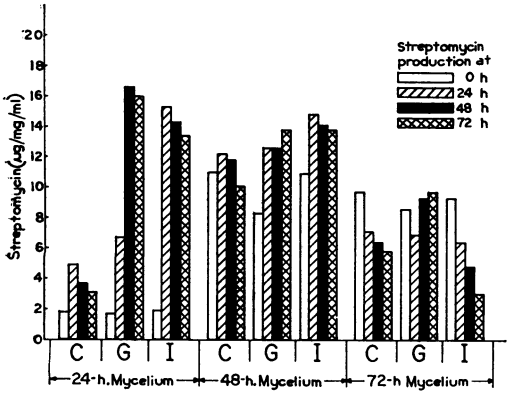

FIG. 1. *Streptomycin production by intact mycelium harvested from fermentation broth of glucose-yeast extract medium. C = without supplement; G = glucose; I = inositol. Glucose and inositol were added in 0.5% final concentration in each culture. Dry weight of mycelium per milliliter of culture was 4.2 mg in the case of 24-hr-old mycelium, 4.35 mg in the case of 48-hr-old mycelium, and 3.85 mg in the case of 72-hr-old mycelium.*

(sometimes even reaching 8.4), slow consumption of glucose, little streptomycin production, and a rapid increase of mycelium. The second phase (24 to 48 hr) was characterized by a gradual decrease in the pH value (sometimes even decreasing to 7.1), rapid and almost complete consumption of glucose, vigorous production of streptomycin, and a further increase of mycelium. The third phase (after 48 hr) was characterized by a second increase in pH value, almost no change in residual glucose, a slight increase and then a decrease of streptomycin, and rapid autolysis of mycelium. When the culture medium was supplemented with calcium chloride, the above-mentioned tendency was almost the same except that streptomycin production increased remarkably during and after the second phase, and the autolysis of the mycelium was delayed (Table 1). Similar relationships were discussed by Hockenhull (1960).

The washed mycelium harvested from the 24-, 48-, and 72-hr-old fermentation broths in glucose-yeast extract medium was suspended in 0.5% glucose solution and in 0.5% inositol solution. The latter compound stimulated streptomycin production of the same strain as was used here in the growing-culture condition in glucose-yeast extract medium (Nomi, 1963). Figure 1 shows streptomycin production by the washed mycelium harvested from the fermentation broth in glucose-yeast extract medium. Figure 2 shows the action of the mycelium harvested from the broths in the same medium as above, but supplemented with 0.02% calcium chloride. In 24-hr-old mycelium, a small amount of streptomycin was left by the washing procedure, and streptomycin production from the supplements in the medium was high. In 48-hr-old mycelium, a large amount of streptomycin was left in the mycelium, and streptomycin production from the supplements was lower than in 24-hr-old mycelium. In 72-hr-old mycelium, the amount of residual streptomycin in the mycelium was still large, and little streptomycin production from the supplements was observed. Thus, the mycelium in three phases showed different activity in producing streptomycin as well as different amounts of streptomycin retained in the mycelium.

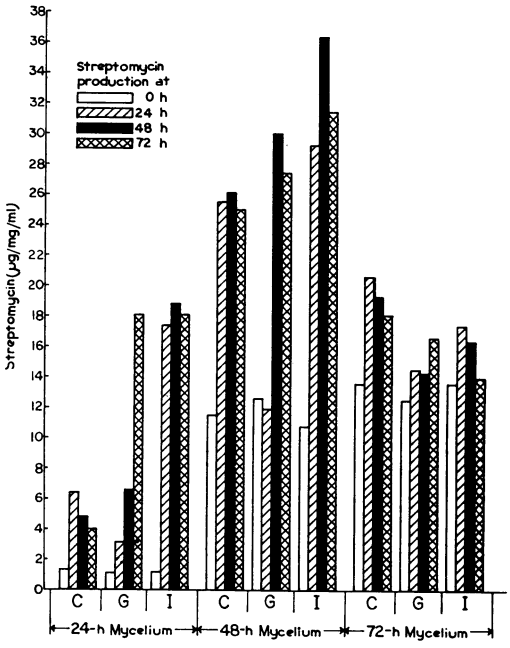

FIG. 2. *Streptomycin production by intact mycelium harvested from fermentation broth of $CaCl_2$-supplemented glucose-yeast extract medium (0.02% $CaCl_2$ added). C = without supplement; G = glucose; I = inositol. Glucose and inositol were added in 0.5% final concentration in each culture. Dry weight of mycelium per milliliter of culture was 3.02 mg in the case of 24-hr-old mycelium, 3.8 mg in the case of 48-hr-old mycelium, and 4.58 mg in the case of 72-hr-old mycelium.*

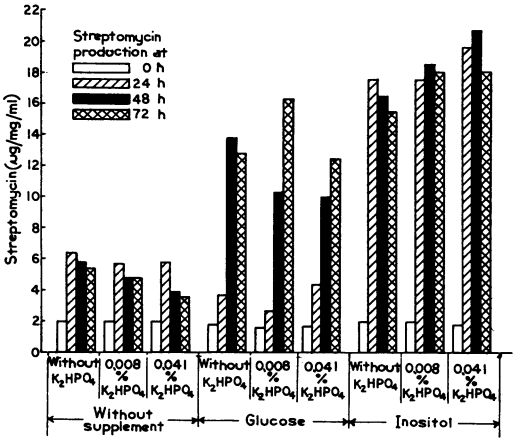

FIG. 3. *Effect of phosphate on streptomycin production by intact mycelium harvested from 24-hr-old fermentation broth of glucose-yeast extract medium. Each concentration of phosphate shows the one which was supplemented in the culture. Glucose and inositol were supplemented in 0.5% final concentration in each culture. Dry weight of mycelium per milliliter of culture was 4.11 mg.*

In the case of the mycelium harvested from the fermentation broth supplemented with calcium chloride, a distinctly different phenomenon was observed: a higher streptomycin production from the precursors in the mycelium, especially in the older material. This phenomenon appears to show that calcium chloride promoted the formation of precursors of streptomycin in the mycelium or the synthesis of the enzyme transforming the precursors to streptomycin.

The effect of calcium ion is reportedly ascribed to precipitation of the excess amount of inorganic phosphate in the medium. It is well known that excess inorganic phosphate interferes with streptomycin production from glucose (Hockenhull, 1960). To rule out this possibility, a small amount of potassium phosphate was added to the suspended culture of washed mycelium (Fig. 3 and 4). A small amount of phosphate ion stimulated streptomycin production in both the 24- and 48-hr-old mycelium. The influence of phosphate on streptomycin production from glucose seems to be more critical than that from inositol. These results show that the mycelium employed in this investigation did not contain excess phosphate to inhibit streptomycin production. Further investigation on the effect of calcium ion was carried out with glucose-yeast extract medium supplemented with various concentrations of $CaCl_2 \cdot 2H_2O$, $CaHPO_4$, and K_2HPO_4 (Table 2). Not only did calcium chloride show a remarkable stimulating effect, but also calcium phosphate stimulated streptomycin production to a high degree, and even a small amount of potassium phosphate stimulated it. It can therefore be concluded that calcium ion has some other effect than the mere precipitation of an excess of phosphate.

Shaw, Henderson, and Seagers (1960) proposed another role for the calcium ion. Calcium was shown to reverse the inhibitory effect of iron on

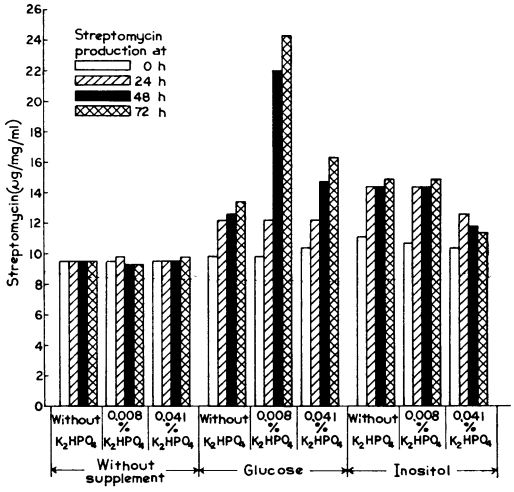

FIG. 4. *Effect of phosphate on streptomycin production by intact mycelium harvested from 48-hr-old fermentation broth of glucose-yeast extract medium. Each concentration of phosphate shows the one which was added to the culture. Glucose and inositol were added in 0.5% final concentration in each culture. Dry weight of mycelium per milliliter of culture was 4.48 mg.*

TABLE 2. *Effect of calcium and phosphate ion on the production of streptomycin in glucose-yeast extract medium*

$CaCl_2 \cdot 2H_2O$	Streptomycin	$CaHPO_4$	Streptomycin	K_2HPO_4	Streptomycin
%	μg/ml	%	μg/ml	%	μg/ml
0	240				
0.01	510	0.01	452	0.013	372
0.02	564	0.02	410	0.026	198
0.05	620	0.05	466	0.064	146
0.1	546	0.1	452	0.128	97
0.2	496	0.2	452	0.256	84

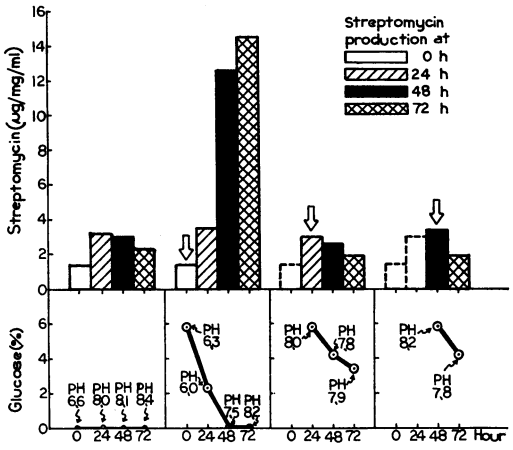

FIG. 5. *Correlation of time of supplementation with glucose to streptomycin formation, pH value, and glucose consumption. Composition of culture: 11 ml of 0.5% NaCl solution and 3 ml of intact mycelium [79.2 mg (dry weight) per 3 ml] in a 50-ml Erlenmeyer flask; 3 ml of 2.83% glucose solution including 0.5% NaCl were added in each supplemented time. Intact mycelium used was harvested from 24-hr-old fermentation broth in glucose-yeast extract medium. Arrows show supplemented time of glucose.*

the synthesis of mannosidostreptomycinase. In the glucose-yeast extract medium, 0.001% ferrous sulfate was added. As some iron is present in yeast extract, the total amount of iron may be more than that necessary for the formation of mannosidostreptomycinase. When the addition of ferrous sulfate to the medium was eliminated, streptomycin production in a growing culture was 300 μg/ml, whereas its production upon the addition of ferrous sulfate was 232 μg/ml, and, with the addition of ferrous sulfate and calcium chloride, 466 μg/ml. This phenomenon will be discussed later.

Figures 1 to 4 show that the high level of streptomycin production from glucose always occurred in or after 48 hr, whereas that from inositol occurred in 24 hr. The optimal time for glucose addition was investigated. Glucose was added at 0, 24, and 48 hr to the suspended culture of washed mycelium harvested from 24-hr-old fermentation broth in glucose-yeast extract medium, and streptomycin production, pH value, and residual glucose were determined. Figure 5 shows that glucose has to be supplemented at the outset of incubation; the pH value of the culture decreased in the first 24 hr and then gradually increased. When the high level of streptomycin production occurred, the pH value was on the alkaline side, and most of the glucose had already been consumed. The various other substances which might have some correlation with streptomycin production were employed as supplements to a suspended culture of washed mycelium (Fig. 6). Three concentrations of each supplement were employed. The substances which are related to the Embden-Meyerhof-Parnas pathway contributed to streptomycin production, but gluconic, glucuronic, and galacturonic acids did not; streptidine and inositol contributed to it, but streptamine and strepturea did not; glucosamine evidently contributed, though glucosaminic acid did only to a lesser degree. The high-level production of streptomycin occurred at an alkaline pH value without exception. Also, a smaller quantity of sugar gave a faster increase in pH and then a faster production to the highest level of streptomycin.

Unless otherwise stated, the only supplement now added to the broth of the mycelium-suspended culture was glucose, and the intact mycelium was harvested from 24-hr-old fermentation broth in glucose-yeast extract medium.

In Fig. 6, the mycelium-suspended culture in 0.5% glucose solution showed, at 24 and 48 hr, a low pH value and, therefore, low streptomycin production; however, it is clear from Fig. 5 that, at this time, a considerable portion of the glucose supplement had already been consumed. This consumption indicates that a part of the glucose consumed was changed to some intermediate substance between glucose and streptomycin, since the streptomycin production of this culture at 72 hr was much higher than that without supplement. The question then arises of whether the intermediate is in the mycelium or in the supernatant. Two kinds of washed mycelium were prepared: one from fermentation broth of glucose-yeast extract medium and the other from the intact mycelium-suspended culture in 0.5% glucose. Two kinds of supernatant were also prepared: one from the same intact mycelium-suspended culture as above, and the other from a glucose-nonsupplemented mycelium-suspended culture. Four cultures were now prepared by the combination of the two kinds of mycelium and two supernatants in 50-ml Erlenmeyer flasks. They were examined for streptomycin formation and were compared with each other and with four control cultures (Table 3). The intact mycelium was not able to accumulate the intermediate substance during the utilization of glucose. Had this accumulation

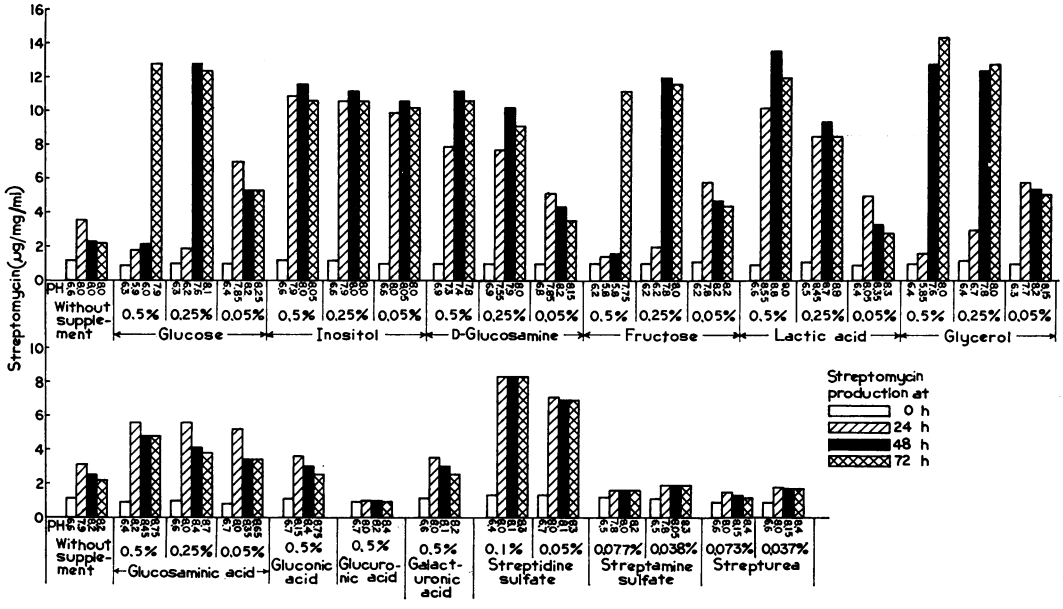

FIG. 6. *Streptomycin production from various substrates by intact mycelium harvested from 24-hr-old fermentation broth of glucose-yeast extract medium. The pH value was determined at the time of each assay of streptomycin. The concentration of each supplement shows the one which was added in each culture. In glucosamine, HCl salt was used and neutralization was carried out before supplementation; each concentration shows that of the acid-free form. Organic acids were neutralized before supplementation. The concentration of organic acid shows that of free acid. Dry weight of mycelium per milliliter of culture was 3.52 mg in the case of glucose, inositol, D-glucosamine, fructose, lactic acid, and glycerol; 3.49 mg in the case of glucosaminic acid, gluconic acid, glucuronic acid, galacturonic acid, streptidine, streptamine, and strepturea.*

occurred, treatments 2, 5, and 7 would have shown a much higher streptomycin production than the corresponding 1, 4, and 6. Treatments 4 and 5 showed about the same streptomycin production as 3. This suggests that almost all the precursor substance, which was derived from the glucose supplement, was retained in the supernatant. Another important aspect of this experiment was that the supernatant prepared from the glucose-supplemented mycelium-suspended culture was able to increase in antibiotic potency without the participation of any mycelium (no. 8 in Table 3). When these results are taken into consideration with pH value and streptomycin production (Fig. 6), the pH value of the supernatant seems to play an important role in transformation of intermediate substance to streptomycin.

To throw further light upon this problem, washed mycelium was added to 0.5% glucose solution, including 0.5% sodium chloride, in an Erlenmeyer flask and shaken for 24 hr. The culture was centrifuged, and the reaction of the supernatant was adjusted with 0.1 N HCl or 0.1 N NaOH solution to pH 4.1, 6.15, 7.0, 8.15, and 9.1. Portions (10 ml) of the supernatant at each pH value were placed in 50-ml Erlenmeyer flasks with a few drops of toluene, and the flasks were placed on a reciprocating shaker. At the same time, other flasks prepared in the same manner were kept in a stationary condition. The streptomycin and the pH level were determined after 24 and 48 hr (Table 4). It was confirmed that the supernatant was able to form streptomycin without the participation of mycelium from an intermediate (or intermediates) which appeared in the supernatant. A much faster increase in antibiotic activity occurred when the supernatant was adjusted to an alkaline pH, whereas a low pH value prevented an increase in antibiotic activity of the supernatant. This seems to explain the necessity for alkaline pH for high streptomycin production (Fig. 6).

The transformation of the intermediate substance to streptomycin requires a slightly alkaline pH, this reaction occurring in the absence of mycelium also. The formation of the intermediate substance from glucose can occur at a lower pH

TABLE 3. *Difference in streptomycin-producing activity between mycelium and supernatant in mycelium-suspended culture*

Treatment	Fresh intact mycelium[a]	Glucose-utilized intact mycelium[b]	Supernatant A[b] (glucose-utilized)	Supernatant B[c] (glucose-nonutilized)	Glucose[d] (2.8%)	NaCl (0.5%)	Streptomycin[e]
	ml	*ml*	*ml*	*ml*	*ml*	*ml*	*μg/ml*
1	3	—	—	—	—	14	10.3
2	—	3	—	—	—	14	6.1
3	3	—	—	—	3	11	68.0
4	3	—	14	—	—	—	78.2
5	—	3	14	—	—	—	60.2
6	3	—	—	14	—	—	33.0
7	—	3	—	14	—	—	15.1
8	—	—	14	—	—	—	54.0

[a] The intact mycelium harvested from 24-hr-old fermentation broth in glucose-yeast extract medium was used.

[b] Intact mycelium harvested from 24-hr-old fermentation broth in glucose-yeast extract medium was suspended in the medium including 0.5% glucose and 0.5% NaCl. After shaking for 24 hr, the culture was centrifuged. The precipitated mycelium was washed with 0.5% NaCl solution and suspended again in a small amount of 0.5% NaCl solution; this was used as glucose-utilized intact mycelium. Dry weight of this suspended mycelium per ml was 22.7 mg. The supernatant was used for supernatant A; pH was 5.8 and streptomycin potency was 14.6 μg/ml.

[c] Intact mycelium harvested in the same manner as above was suspended in the medium including only 0.5% NaCl (without glucose). After shaking for 24 hr, the culture was centrifuged and the supernatant was used as supernatant B; pH was 8.0 and streptomycin potency was 15.5 μg/ml.

[d] Glucose was dissolved in 0.5% NaCl solution.

[e] All combinations in 50-ml flasks were kept on a reciprocating shaker for 3 days. The maximal production of streptomycin of each combination is presented in this column.

TABLE 4. *Effect of pH value on streptomycin formation in the supernatant of mycelium-suspended culture*

Adjusted pH value of supernatant*	0 hr	With shaking				Without shaking			
		24 hr		48 hr		24 hr		48 hr	
	Streptomycin†	Streptomycin	pH	Streptomycin	pH	Streptomycin	pH	Streptomycin	pH
4.1	6.4	1.7	4.4	1.8	4.4	1.6	4.1	1.8	4.4
6.15	8.0	14.2	6.8	58.2	6.8	13.0	6.6	30.0	6.7
7.0	8.0	49.0	7.2	61.8	7.4	29.0	7.0	51.5	7.5
8.15	8.0	60.8	7.4	60.9	7.6	56.4	7.1	57.8	7.5
9.1	10.4	57.7	7.6	58.4	7.7	54.6	7.7	56.9	7.3

* An 11-ml amount of intact mycelium [21.4 mg (dry weight) per ml] harvested from 24-hr-old fermentation broth in glucose-yeast extract medium was added to 40 ml of 0.638% glucose solution in a 250-ml Erlenmeyer flask. After shaking for 24 hr, the culture was centrifuged, and the supernatant (pH 6.15) was adjusted to each pH value.

† Streptomycin produced is expressed as micrograms per milliliter.

value than 7.0 but needs the presence of mycelium. Another important fact observed in Table 4 was that, at pH 8.15 and 9.1, no difference was found between shaking and stationary conditions in the conversion of the intermediate substance to streptomycin, while at pH 7.0 and 6.15 a shaking condition was preferred.

To gain more insight into the characteristics of the supernatant of the mycelium-suspended culture, other experiments were carried out with the same supernatant used above. The supernatant at pH 6.15 and 8.15 was heated in a steam bath (100 C) for 10 min and placed on a reciprocating shaker. No increase in the quantity of streptomy-

cin occurred. In fact, it decreased from 8.0 to 2.0 μg/ml after steam treatment (Table 7). These results suggest that the supernatant included some heat-labile substance which was necessary for an increase of streptomycin.

The same supernatant (pH 7.0 and 8.15) as used in the above experiment was treated with cation-exchange resin (Amberlite IRC-50). An 80-ml portion of each supernatant was treated separately with 12 ml of the resin which had been equilibrated with 0.2 M phosphate buffer solution (pH 7.0) or with saturated sodium bicarbonate solution. A 10-ml portion of the supernatant which came through the resin was put into a 50-ml Erlenmeyer flask and placed on a reciprocating shaker. As shown in Table 5, the resin which was equilibrated at pH 7.0 adsorbed the entire antibiotic potency and prevented almost all increase in antibiotic activity. However, in the case of the resin which was equilibrated with saturated sodium bicarbonate solution, the supernatant which came through the resin still had the same activity as that of the untreated supernatant. According to Doery, Mason, and Weiss (1950), the latter resin should adsorb streptomycin; Table 5 shows that authentic streptomycin was adsorbed to this resin completely. These results suggest that the above-postulated intermediate substance in the supernatant has a lower basic potency than streptomycin.

The increasing antibiotic activity of the supernatant was inactivated at pH 4.1 (Table 4). This phenomenon seems to suggest that the above-mentioned intermediate substance was not stable at low pH values. However, if some enzyme is necessary for the transformation of the intermediate substance to streptomycin, this phenomenon can also be interpreted as an inactivation of the enzyme. If this inactivation at a low pH value were reversible, streptomycin would increase again when the pH value is brought back to the alkaline side. The supernatant was adjusted at pH 4.0 and shaken for 24 hr; then the pH value was adjusted at 8.0, and the supernatant was placed on a reciprocating shaker for 48 hr. As shown in Table 6, no increase in antibiotic potency occurred. This reaction is, therefore, irreversible. However, the intermediate substance was not completely destroyed at pH 4.0. As shown in Table 7, the inactivated supernatant was able to form the same amount of streptomycin as the active supernatant by the addition of fresh intact mycelium, which is considered to have the necessary enzyme systems. Furthermore, the supernatant which was inactivated by steam treatment was also able to form the same amount of

TABLE 5. *Adsorption of the activity in producing streptomycin in the supernatant of mycelium-suspended culture by ion-exchange resin (Amberlite IRC-50)*

Adjusted pH value of supernatant[a]	Streptomycin[b] before treatment with resin	After treatment with resin[c]		After shaking of treated supernatant			
				24 hr		48 hr	
		Streptomycin	pH	Streptomycin	pH	Streptomycin	pH
7.0	8.0	0	7.0	0	7.2	0.5	7.8
8.15	8.0	4.4	8.4	20	7.6	19.0	8.0
Streptomycin sulfate solution[d]							
In distilled water	21.0	0					
In 0.5% NaCl solution	21.6	0					

[a] The same supernatant as that in Table 4.

[b] Streptomycin produced is expressed micrograms per milliliter.

[c] Amberlite IRC-50 (12 ml) was applied to 80 ml of supernatant. For treatment of pH 7.0 and pH 8.15 supernatants, resin was equilibrated with phosphate buffer solution (pH 7.0, 0.2 M) and saturated sodium bicarbonate solution, respectively.

[d] Streptomycin sulfate was dissolved in distilled water and 0.5% NaCl solution. Immediately after dissolution, 80 ml of each solution were treated with 12 ml of resin equilibrated with saturated sodium bicarbonate solution.

streptomycin as the active supernatant by the addition of fresh intact mycelium (Table 7).

TABLE 6. *Influence of low pH value on the activity in producing streptomycin in the supernatant* of mycelium-suspended culture*

Conditions	Streptomycin	pH
	μg/ml	
Before shaking	4.0	4.0
After shaking		
24 hr	1.5	4.1
48 hr	1.6	4.1
0 hr†	1.5	8.0
24 hr	1.3	7.25‡
48 hr	1.7	7.45

* This supernatant was prepared separately from, but by the same manner as, that in Table 4, and was used in this experiment after adjustment to pH 4.0. This supernatant in its original state at pH 5.6 contained 6.1 μg/ml of streptomycin; by shaking at pH 8.0 for 48 hr, streptomycin increased to 57.6 μg/ml.

† After 24 hr of shaking, the pH was readjusted to 8.0, and the supernatant was shaken again.

‡ After the second 24 hr of shaking, the pH was again adjusted to 8.0.

DISCUSSION

When calcium chloride was added to glucose-yeast extract medium, a remarkable stimulation of streptomycin production occurred in the growing culture, as well as in a mycelium-suspended culture. As calcium chloride is able to reverse the inhibitory effect of iron on the synthesis of mannosidostreptomycinase, a part of the above-mentioned stimulation of streptomycin production by calcium chloride might be attributed to the increased mannosidostreptomycinase. However, it remains to be determined whether the difference between Fig. 1. and Fig. 2 was caused only by the cleavage of mannosidostreptomycin. If this is true, the interpretation of this phenomenon suggests that the mycelium grown in a calcium-supplemented medium includes considerably large amounts of mannosidostreptomycinase as well as mannosidostreptomycin, while the mycelium grown in calcium-nonsupplemented medium did not include either mannosidostreptomycinase or mannosidostreptomycin, or both. Furthermore, it is important in this case that the mannosidostreptomycin be considered a precursor of streptomycin. Other effects of calcium chloride are noted in Table 1. When calcium chloride was added to the medium, the pH value

TABLE 7. *Streptomycin formation by intact mycelium from the intermediate substance included in the supernatant[a] which was inactivated at low pH value and by steam treatment*

Conditions	Supernatant adjusted to pH 4.1		Supernatant adjusted to pH 8.15		Treated with steam[c]				Control[d] (0.5% NaCl solution including 0.18% glucose)	
					Supernatant at pH 6.15		Supernatant at pH 8.15			
	Streptomycin[b]	pH	Streptomycin	pH	Streptomycin	pH	Streptomycin	pH	Streptomycin	pH
Before shaking	6.4		8.0		2.2		2.2			
After shaking for 24 hr.	1.7	4.4	60.8	7.4	2.0	6.6	2.1	7.4		
After addition of intact mycelium[e]										
0 hr	9.7	5.8	49.6	6.8	9.0	6.3	9.0	6.5	3.1	6.2
24 hr	99.2	7.4	106.4	7.6	97.0	7.6	90.0	7.6	42.4	7.4

[a] The same supernatant as in Table 4 was used.

[b] Streptomycin produced is expressed as micrograms per milliliter.

[c] Supernatants of pH 6.15 and 8.15 were heated by steam (100 C) for 10 min.

[d] As the supernatant used here included 0.18% residual glucose, the production of streptomycin from this amount of glucose by intact mycelium was determined as the control.

[e] After 24 hr of shaking, intact mycelium harvested from 24-hr-old fermentation broth in glucose-yeast extract medium was added to 9.5 ml of supernatant. Shaking was then continued.

was lower after the first phase and the autolysis of the mycelium was delayed, compared with the calcium-nonsupplemented broth. These phenomena suggest some other effect of calcium ion on streptomycin production, directly or indirectly, than the above-mentioned reason. It should be pointed out that the effect of calcium chloride on streptomycin formation depends on the culture medium; in glucose-meat extract-peptone medium, the addition of calcium chloride produced only a slight effect with the same strain.

Concerning the metabolism of glucosamine, two pathways have been recognized in microorganisms (Imanaga et al., 1960): (i) the transformation to fructose-6-phosphate through glucosamine-6-phosphate and its deamination, and (ii) the formation of glucosaminic acid by oxidation without phosphorylation. Further degradation of glucosaminic acid is not usual, but it can be converted to ammonia and 2-keto-3-deoxygluconic acid by bacteria. So far, no metabolism of glucosamine by *S. griseus* has been reported in connection with streptomycin production. As noted in Fig. 6, glucosamine obviously contributed to streptomycin formation, as also did glucosaminic acid, but in a lesser degree. Though the incorporation of glucosamine into the streptomycin molecule is not shown in this study, Hockenhull (1960) proposed the mechanism of ring closure of D-glucosamine-6-phosphate to scyllo-inosamine as the first step in the formation of the streptidine moiety. According to Romano and Sohler (1956), and Sohler, Romano, and Nickerson (1958), hexosamine is one of the main components of the cell wall of *S. griseus*. The study of the metabolism of glucosamine would seem to be important for the understanding of streptomycin production.

When the intact mycelium was suspended and shaken in glucose solution for 24 hr, the streptomycin-producing activity appeared in the supernatant rather than in the mycelium (Table 3). The activity of the supernatant was inactivated at pH 4.0 to 4.1 and by heat (100 C, 10 min). It seems reasonable to assume that the low pH- and heat-labile substance is an enzyme and that the intermediate substance itself has no antibiotic activity; the increase of antibiotic activity of the supernatant at the slightly alkaline pH value was mediated by the transformation of the intermediate substance to streptomycin by the action of the enzyme. If this is true, all of the weak antibiotic activity, which was included in the supernatant, has to be attributed to the already formed streptomycin. This streptomycin should be adsorbed completely to Amberlite IRC-50 equilibrated with saturated sodium bicarbonate solution. This was not the case, however; the antibiotic potency of the supernatant was only partially adsorbed, and the supernatant, which came through the resin, still increased its antibiotic activity after shaking. Furthermore, the antibiotic activity of the supernatant decreased by about 20 to 30% immediately after the pH was adjusted to 4.1 to 4.0 at room temperature (Tables 4 and 6). Streptomycin itself is stable at this pH value. Thus, it is clear that all of the antibiotic activity of the supernatant was not represented by the streptomycin. A part of the antibiotic activity in the supernatant (or the major part of it) has to be attributed to an intermediate substance; that is, the intermediate substance has some weak antibiotic activity. Inactivation of the active supernatant at the low pH value apparently involves the inactivation of the biologically active intermediate substance. The inactivation of the active supernatant at the low pH value was irreversible; this may mean that the inactivation of the active intermediate was irreversible. However, the inactivated intermediate substance was able to form the same amount of streptomycin as that formed from active intermediate substance by intact mycelium; this means that the inactivated intermediate substance requires some enzyme to be restored to original form.

Although the above-mentioned biologically active intermediate substance has a weak antibiotic activity and remarkably increases in it at a slightly alkaline pH value, it is still not clear whether the enzyme effects the transformation of the active intermediate substance to streptomycin. The inactivation of the supernatant at the low pH value or by heat does not necessarily mean the inactivation of only the active intermediate substance.

Regardless of the ambiguity concerning the action of the enzyme, it is clear from the experiment with cation-exchange resin (Table 5) that the reaction transforming the active intermediate substance to streptomycin was carried out in such a manner that it was made more basic. This reaction is thought to occur at a late stage and to comprise a very few steps of the biosyn-

thetic pathway from glucose to streptomycin, because the intermediate substance already has a weak antibiotic activity and is easily transformed to streptomycin merely by a change in pH.

It is important that the above-mentioned reaction requires a slightly alkaline pH value, whereas the intermediate substance is able to be produced at a lower pH value. This is thought to be one of the reasons that a slightly alkaline fermentation broth is necessary for high streptomycin production. The precursor of streptomycin which was observed in high concentration in 48-hr-old intact mycelium (Fig. 2) might be identical or very similar to the above-mentioned intermediate substance.

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