consumption or yield of products. Increase in carrier gas (hydrogen) from 5 to 20 liters/hour reduced consumption but not the rate of decay of catalyst activity.

Consumption fell from 73 to 66% at 325° after the passage of 16 moles (1632 g.) of alcohol. At 350° consumption, originally 90%, was halved after the passage of 12 moles. At higher temperatures consumption fell off more rapidly, thus at 520° it fell to 20% after the passage of only 3 moles of alcohol.

Acrolein was detectable at 390° using hydrogen as carrier gas (5 liters/hour). The product, b. p. above 160°, at various temperatures contained the following amounts of tetrahydrofurfuryl alcohol: 67.1 (325°), 66.8 (350°), 66.8 (400°), 64.7 (450°), 65.0 (490°) and 63.0% (520°).

Reduction of pressure lowered consumption. At 8 mm. consumption (input 0.5 mole/hour) was 65% and yield of dihydropyran 61%.

Summary

1. 2,3-Dihydropyran has been shown to split into acrolein and ethylene on heating the vapor to temperatures in the region 400–500°.

2. Tetrahydrofurfuryl alcohol is readily dehydrated at low temperatures over Alumina (250–300°) to give dihydropyran, but acrolein is not formed at high temperatures since the dihydropyran first formed is dehydrated further before it can split.

3. An aluminum silicate catalyst can be used at high temperatures to convert tetrahydrofurfuryl alcohol directly into acrolein.

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF WISCONSIN]

Streptolin, a New Antibiotic from a Species of Streptomyces[1]

BY R. W. RIVETT AND W. H. PETERSON

Actinomycetes have been the source of several antibiotics, among which the most important are streptothricin[1a] and streptomycin.[2] In the course of an extensive survey of actinomycetes isolated from soil, a culture has now been found which is capable of producing in submerged fermentations an antibacterial substance, highly active against both gram-negative and gram-positive bacteria. This culture has been designated *Streptomyces* No. 11 and the antibiotic isolated from its culture filtrates has been named *streptolin*. Some of the factors affecting the production of streptolin by *Streptomyces* No. 11 were investigated and the chemical, physical and biological properties of streptolin studied.

Fermentations were carried out in flasks shaken continuously in order to find a suitable medium for the production of streptolin. A soybean meal–glucose medium (medium B, Table I) allowed the formation of the highest yields of streptolin in shaken flasks. Medium A was used in larger scale production with very good results, but gave only 7500 units[3] per ml. in shaken flasks. A nitrate–glucose synthetic medium, C, produced 10,400 units per ml. (Table I).

For the production of larger batches of fermented medium suitable for the isolation of streptolin, 30-liter laboratory fermenters were employed. The highest yields, 48,000 units per ml., were obtained on medium A, Table II.

TABLE I

PRODUCTION OF STREPTOLIN IN SHAKEN FLASKS

	Media	Max. yield, units/ml.	Age, hr.
A.	1% soybean meal, 1% corn steep (solids), 1% glucose, 0.5% NaCl, 0.1% $CaCO_3$	7,500	70
B.	2% soybean meal, 0.5% Curbay BG, 2% glucose, 1% NaCl, 0.1% $CaCO_3$	35,000	72
C.	0.05% NH_4NO_3, 0.5% $NaNO_3$, 0.05% $MgSO_4 \cdot 7H_2O$, 0.005% $FeSO_4 \cdot 7H_2O$, 1% glucose, 0.1% $CaCO_3$, 0.6% K_2HPO_4[a]	10,400	77

[a] Sterilized phosphate was added to the other ingredients after sterilization bringing the *p*H to 8.

Medium D, differing only slightly from medium B, Table I, gave about 75% of the yield obtained in shaken flasks. A synthetic medium, E, gave approximately the same yield as was obtained in flask cultures on medium C.

TABLE II

PRODUCTION OF STREPTOLIN IN 30-LITER FERMENTERS

	Media	Max. yield units/ml.	Age, hr.
A.	1% soybean meal, 1% corn steep (solids), 1% glucose, 0.5% NaCl, 0.1% $CaCO_3$	48,600	70
D.	2% soybean meal, 0.25% Curbay BG, 2% glucose, 1% NaCl, 0.1% $CaCO_3$	27,300	65
E.	0.3% NH_4NO_3, 1% glucose, 0.3% NaCl, 0.05% $MgSO_4 \cdot 7H_2O$, 0.005% $FeSO_4 \cdot 7H_2O$, 0.1% $CaCO_3$ and 0.5% K_2HPO_4[a]	8,900	93

[a] Added after sterilization bringing *p*H to 8.

In an effort to increase the yield of streptolin on Medium B in the fermenters, a series of runs were made in which agitation and aeration were

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(1a) Waksman and Woodruff, *Proc. Soc. Exptl. Biol. Med.*, **49**, 207 (1942).

(2) Schatz, Bugie and Waksman, *ibid.*, **55**, 66 (1944).

(3) A unit of streptolin was defined as that amount which when dissolved in one ml. of 0.75% peptone, 0.25% yeast extract broth at *p*H 7.2 would just inhibit the growth of *Escherichia coli* H52 during an eighteen–twenty hour incubation at 37°, when the inoculum was 0.00005 ml. of a twenty-hour broth culture per ml.

varied. The results (Table III) show that there is an optimum aeration rate at about 0.25 volume of air per min. per volume of medium with an agitation rate of 195 r. p. m. Both increasing and decreasing aeration or agitation from this optimum resulted in a decrease in yield.

TABLE III

EFFECT OF AGITATION AND AERATION ON STREPTOLIN PRODUCTION IN 30-LITER FERMENTERS[a]

Agitation r. p. m.	Aeration vol./vol./min.	Max. yield units/ml.	Age, hr.
70	0.25	500	58
195	.66	23,900	60
195	.25	27,300	65
195	.25	26,400	60
375	.25	23,400	83
375	.66	15,300	83

[a] Medium D, Table II.

Some of the chemical changes occurring in a fermentation on a synthetic medium plus casein hydrolysate are given in Fig. 1. The main production of streptolin occurred after most of the glucose had been used and during the period of most rapid non-ammonium-nitrogen utilization and growth. A rise in *p*H probably due to ammonia liberation also occurred at the time of streptolin formation. It was found that soybean meal and corn steep liquor were the best nitrogen sources, while glucose and glycerol were the best carbon sources. Sodium chloride was essential for high yields of streptolin on media containing soybean meal.

Streptolin was isolated from clarified beer by successive adsorptions on and elutions from Filtercel and Darco G-60. The material obtained by ether precipitation from the final eluate was readily dried to a tan, hygroscopic powder having an activity of 22,000 to 33,000 units of streptolin per mg. The over-all recovery was generally of the order of 60–70%.

Further purification was accomplished by conversion of the crystalline helianthate to its hydrochloride by a method similar to that described for the conversion of streptothricin helianthate to streptothricin hydrochloride.[4] Streptolin hydrochloride purified in this manner had an activity of 34,000 units per mg. or 97% of the theoretical activity of pure hydrochloride, calculated on the basis of the activity and helianthine content of the crystalline helianthate.

Solutions of streptolin exhibit maximum thermal stability at *p*H 3 to 3.5, being about 50% inactivated when held at 120° for one hour. It is much less stable in more alkaline or more acid solutions. Titration of streptolin hydrochloride indicates that the molecule has more than one basic group buffering in the range of *p*H 7.5 to 9.5.

The results of a comparison of some of the chemical, physical, and antibacterial properties of the hydrochlorides and crystalline helianthates of streptolin, streptomycin, and streptothricin are given in Table IV. Streptolin differs markedly from streptomycin in the nitrogen content of the helianthates and in the maltol test.[5] Streptolin was similar to streptothricin in chemical properties, but the two exhibited considerable differences in physical and antibacterial properties. Streptolin had a lower optical rotation and was more readily adsorbed on Filter-Cel. In addition, streptolin was six times as active as streptothricin in a broth assay while it was only 0.07 as active in an agar diffusion assay. This may be considered evidence for the low rate of diffusion of streptolin through agar.

A bacterial spectral comparison of the three antibiotics was made on sixty cultures of bacteria by the dilution method. A portion of these spectra illustrating the differences between streptolin and the other antibiotics are recorded in Table V. The ratios of inhibiting concentrations of streptolin to streptothricin and streptolin to streptomycin for each test organism are included in order to facilitate comparisons. If any two of the antibiotics were identical, the ratios would be constant

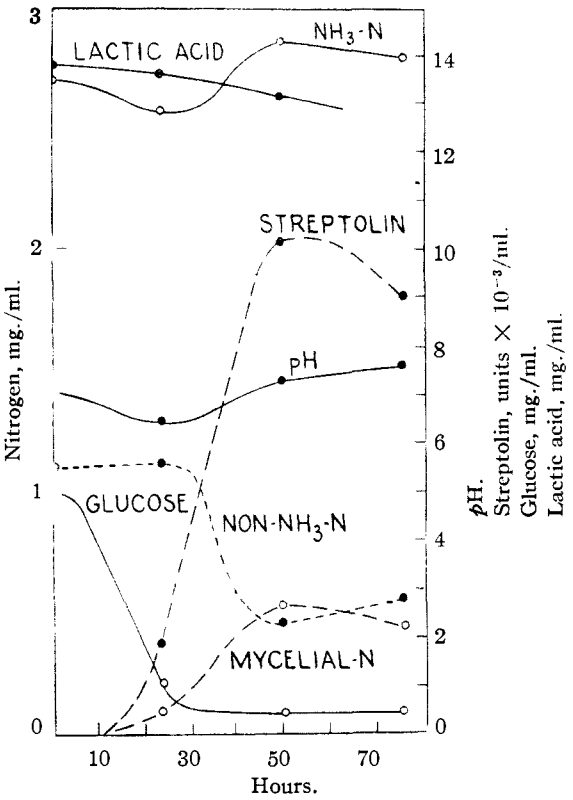

Fig. 1.—Chemical changes occurring in a *Streptomyces* No. 11 fermentation. Medium: 1.75% ammonium lactate, 0.5% glucose, 1.0% hydrolyzed casein, 0.6% KH_2PO_4, 0.3% NaCl, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% $CaCO_3$, 0.005% $FeSO_4 \cdot 7H_2O$.

(4) Peck, Walti, Graber, Flynn, Hoffhine, Allfrey and Folkers, THIS JOURNAL, **68**, 772 (1946).

(5) Schenck and Spielman, *ibid.*, **67**, 227 (1945).

TABLE IV

COMPARISON OF PROPERTIES OF STREPTOLIN, STREPTOMYCIN AND STREPTOTHRICIN

	Streptolin	Streptomycin	Streptothricin
M. p., °C., of helianthate (uncor.)	207–211 (dec.)	205–208 (dec.)	207–210 (dec.)
Nitrogen, helianthate,[a] %	12.69	11.77	12.81
Helianthine in helianthate,[b] %	63.6	62.9	63.6
Activity, helianthate,[c] units/mg.	15,400	...	2500
Maltol, hydrochloride	Negative	Positive	Negative
$[\alpha]_D$, hydrochloride	−22° (*c*, 1.8), −25°(*c*, 2.9)	−84° (*c*, 0.5)[d]	−51.3 (*c*, 1.4)[h]
Filter-Cel adsorption,[e] % ads.	99	...	51
Activity, hydrochloride,[f] units/mg.			
E. coli turb. assay	33,000	...	5,500
B. subtilis plate assay	33,000	...	460,000
Activity/α_D ratio[g]	-1.5×10^6	...	-0.16×10^6

[a] By Kjeldahl method which gives low values on azo compounds. [b] Determined colorimetrically. [c] Streptolin units, turbidimetric method. [d] Kuehl, Peck, Walti and Folkers, *Science*, **102**, 34 (1945). [e] Aqueous solutions of hydrochlorides having equal activity in the streptolin turbidimetric assay, treated with 5% Filter-Cel at *p*H 7.5. [f] Streptolin used as standard. Results in streptolin units. [g] Activity of solutions of hydrochlorides in streptolin units per ml. (turb. assay) divided by rotation of plane-polarized light in degrees per decimeter. [h] Peck, *et al.*, THIS JOURNAL, **68**, 773 (1946).

throughout within limits of experimental error. The differences between the highest and lowest ratios are sufficiently great to warrant the conclusion that streptolin is not identical with either streptomycin or streptothricin. However, as in the case of chemical and physical properties, streptolin appears to be more closely related to streptothricin than to streptomycin.

TABLE V

COMPARATIVE BACTERIAL SPECTRA OF STREPTOLIN, STREPTOTHRICIN AND STREPTOMYCIN[a]

Test organism	Inhibiting concentration units/ml.[b] Streptolin	Streptothricin	Streptomycin	Ratios of inhibiting concn. Stl/Sth	Stl/Stm
Aer. aerogenes	3.3	0.1	0.1	33	33
Aer. polymyxa	1.0	1.0	.3	1.0	3.3
Bac. cereus	3.3	3.3	.1	1.0	33
Bac. fusiformis	0.1	0.06	10.0	1.6	0.01
Bac. megatherium	.3	.01	0.03	30	10
Bac. mycoides	10.0	1.0	.03	10	333
E. coli H52	1.0	0.1	.1	10	10
Pr. vulgaris	3.3	1.0	.1	3.3	33
Ps. aeruginosa	10.0	0.3	.3	33	33
Ps. fluorescens	33.0	1.0	1.0	33	33
Isolate no. 11[c]	> 100	0.5	...	> 200	...
Isolate no. 14[c]	> 1000	2.0	...	> 500	...
Isolate no. 17[c]	> 1000	6.0	...	> 167	...
Isolate no. 23[c]	6.0	6.0	...	1.0	...

[a] Dilution assay in following medium: 0.75% peptone, 0.25% yeast extract. *p*H 7.2. Inoculum: 1 drop twenty-four hour broth culture per 10 ml. [b] Streptolin (stl) expressed in streptolin units, streptothricin (sth) and streptomycin (stm) as per units on labels from Merck and Co. and Abbott Laboratories, respectively. [c] Streptolin-resistant bacteria isolated from natural sources by plating on media containing high concentrations of streptolin.

Toxicity tests with mice showed that streptolin is toxic at a level of 8–10 mg. per kilo of body weight when injected intravenously. Deaths occurred four to five days after injection. Results of the toxicity tests are given in Table VI.

Since streptolin diffuses poorly through agar, a cup-plate method of assay was not feasible. Dilution assays proved to be tedious and undependable. It was found that a turbidimetric assay with *E. coli* H52 offered a rapid, accurate method for estimation of streptolin in culture filtrates as well as in various fractions occurring during the isolation procedures. This assay was used throughout the work.

TABLE VI

TOXICITY OF STREPTOLIN TO MICE[a]

Mg./mouse	No. of mice	Deaths	Av. survival time, days
0.09	11	0	..
.13	10	4	6
.20	11	7	5
.27	5	5	4

[a] Composite table of results obtained at the University of Wisconsin, Abbott Laboratories, and The Upjohn Company. Crude streptolin containing 30,000 units per mg. was injected intravenously. Mice weights averaged 20 g.

Experimental

Fermentations.—The spores of *Streptomyces* No. 11 were preserved on dried soil. A transfer of these to an agar medium consisting of 1% dextrin, 0.3% corn steep liquor, 0.5% tryptone, 0.2% dipotassium hydrogen phosphate, 0.2% sodium chloride, and 0.001% iron resulted in heavy growth and sporulation after incubation at 25° for five days. Five hundred-ml. Erlenmeyer flasks containing 100 ml. of 1% corn steep liquor (dry basis), 1% soybean meal, 1% glucose and 0.15% calcium carbonate were inoculated with 1 to 2 ml. of a spore suspension of *Streptomyces* No. 11. After incubation on a reciprocating shaker at 25° for sixty to seventy-two hours, the culture was ready for use as vegetative inoculum. Five per cent. vegetative inoculum was used in all fermentations.

Flask fermentations were carried out in 500-ml. Erlenmeyer flasks containing 100 ml. of medium. The flasks were incubated at 25° on a reciprocating shaker with a 4-inch stroke making 80–90 cycles per minute. Samples from the flasks were assayed for streptolin by the *E. coli* turbidimetric method on the second, third, and fourth days of incubation.

Fermentations in 30-liter fermenters were done at 25° with 15 liters of medium in each fermenter. This equipment will be described in detail elsewhere.

Isolation of Streptolin.—Four 30-liter fermenter runs were harvested at ninety-four hours and the pooled cultures were acidified to *p*H 2.5 with sulfuric acid and treated with 2 kg. of Celite 545. The mycelium and filter aid were removed in a filter press. The beer was ad-

justed to *p*H 7.9 and a flocculent precipitate removed by filtration through a Celite cake. Fifty liters of clarified beer at *p*H 7.9 containing 26,000 units of streptolin per ml. was passed through 2 kg. of Filter-Cel (a diatomaceous earth, Johns-Manville Co.) previously laid down in the press. Seventy-six per cent. of the streptolin was adsorbed in this step. After thorough washing of the Filter-cel adsorbate with water, 96% of the streptolin was eluted by the passage of 16 liters of 7.5% pyridine hydrochloride at *p*H 1.5 through the cake.

A portion of the pyridine hydrochloride eluate, 13 liters containing 65,000 units per ml., was passed through a cake of 475 g. of activated carbon, Darco G-60, at *p*H 2.3. Eighty eight per cent. of the streptolin was adsorbed on the carbon. After washing the cake with water, 93% of the adsorbed streptolin was eluted by passing 6 liters of 0.03 *N* hydrochloric acid in 50% ethyl alcohol through the cake.

A 4-liter portion of the acid alcohol eluate containing 145,000 units per ml. was concentrated *in vacuo* to 700 ml. The solution was adjusted to *p*H 7.6 with sodium hydroxide and again concentrated *in vacuo* to remove the pyridine. Sodium chloride crystallized from the resulting sirup and was removed by centrifugation after the addition of two volumes of methanol. Addition of five volumes of ethyl ether resulted in the precipitation of streptolin hydrochloride as a gummy mass. The precipitate was dried *in vacuo* at 45° and the resulting product was broken up to give 17 g. of a tan hygroscopic powder, 32,300 units per mg.

Streptolin Helianthate.—A solution of 1.2 g. of streptolin hydrochloride in 25 ml. of water at *p*H 6.7 was treated with 1.25 g. of methyl orange in 35 ml. of hot water. After cooling and standing for two hours at 4° the dark red precipitate was removed by filtration, washed with 40 ml. of water and dried; yield was 1.69 g. The helianthate was crystallized from methanol and dried in a vacuum oven. The crystals had an activity of 15,200 units of streptolin per mg.; Kjeldahl nitrogen, 12.69%; helianthine, 63.6%; and m. p. 207–211° with decomposition.

By the method[4] described for the conversion of streptothricin helianthate to its hydrochloride, 240 mg. of streptolin helianthate was converted to yield 55 mg. of streptolin hydrochloride, 34,000 units per mg.; $(\alpha)^{28}D$ −22° (*c*,1.8).

***E. coli* Turbidimetric Assay for Streptolin.**—Into 20 × 150 mm. test-tubes in copper racks standard and unknown streptolin solutions were pipetted in amounts not exceeding 0.3 ml. per tube. The standard series consisted of 10 tubes containing 0.07, 0.06, 0.055, 0.05, 0.045, 0.04, 0.035, 0.03, 0.025 and 0.02 ml. of standard streptolin solution, 10,000 units per ml. For each unknown sample 5 tubes containing graded levels of sample with an estimated 250 to 600 units of streptolin per tube were set up. A 14 ml. portion of 1% tryptone, 0.5% yeast extract and 0.5% beef extract at *p*H 7.3–7.4 previously inoculated with 2 ml. per 100 ml. of a twelve to twenty-hour broth culture of *Escherichia coli* H52 was then pipetted into each tube. The rack of tubes was covered with a sheet of copper and incubated in a water-bath at 37° for three hours.

The extent of growth in each tube was measured turbidimetrically with either an Evelyn photoelectric colorimeter (660 mμ filter) or a Lumetron colorimeter (640 mμ filter). Galvanometer readings plotted against units of streptolin per tube yielded a standard curve such as the one shown in Fig. 2. The amount of streptolin in each unknown tube was read from the standard curve. When several levels of an unknown solution gave galvanometer readings within the range of the standard curve, the calculated potencies checked each other within 5%. Generally, not more than a 10% variation was noted in assays of the same solution on different days.

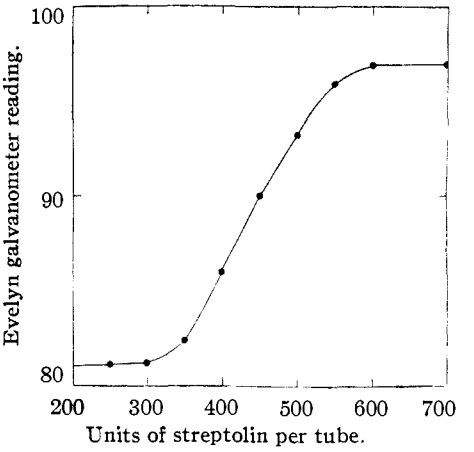

Fig. 2.—Typical standard curve for streptolin turbidimetric assay.

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Summary

Production in submerged culture of streptolin, a new antibiotic from a species of Streptomyces, has been studied with respect to media composition and aeration.

Streptolin has been isolated as the hydrochloride and a crystalline derivative (helianthate) has been obtained.

In physical, chemical and antibacterial properties streptolin is quite similar to streptomycin and streptothricin. It is rather toxic to mice.

Streptolin resembles streptomycin and streptothricin in certain chemical and antibacterial properties, but differs markedly from these antibiotics in other respects, *viz.*, specific rotation of the hydrochloride, adsorption on Filter-Cel, antibacterial activity of the helianthate and hydrochloride.[6]

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(6) Workers at the Upjohn Company have confirmed an antibiotic which has many properties in common with our antibiotic. Final decision on the identity or non-identity of the two compounds must await further comparisons on compounds of known purity.