TETRAMYCIN, A NEW POLYENE ANTIBIOTIC

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A new polyene antibiotic was isolated from the culture broth of a new *Streptomyces*, designated as *Streptomyces noursei* HAZEN *et* BROWN, 1950 var. *jenensis* nov. var. JA 3789. This antibiotic is a tetraene and was named tetramycin. The morphological and physiological characteristics of *Streptomyces* JA 3789 and the isolation, purification, physiochemical, and biological properties of the antibiotic are described. Tetramycin was differentiated from related antibiotics by physical and chemical data.

In the course of screening for new polyene antibiotics[1], we have obtained a new tetraene antibiotic in crystalline form from the fermentation broth of a new *Streptomyces* culture, which, according to detailed taxonomic studies, must be considered a variety of *Streptomyces noursei*. The name *Streptomyces noursei* HAZEN *et* BROWN, 1950 var. *jenensis* nov. var. JA 3789 was proposed for the organism. The strain producing this antibiotic was isolated from a sample of humus containing loam collected in Vietnam. The antibiotic, which was named tetramycin, is active against yeasts and fungi.

In this report, the characteristics of the organism, the medium for antibiotic production, the isolation and some of the physical, chemical and biological properties of the antibiotic are presented.

Characteristics of the Strain

The morphological properties of the strain are summarized as follows:

Sporophores: Spirals as side branches of long aerial hyphae.
Spores: With spiny surface.
Color of aerial mycelium: White turning light grey to brownish-grey.
Color of the substrate mycelium: Colorless to pale yellow to faint ochre, sometimes with a tinge of olive green.

The cultural characteristics of the strain on various media and the physiological characteristics are listed in Tables 1 and 2, respectively. The utilization of carbon sources by the organism is shown in Table 3.

The results of these studies are as follows:

Comparison of *Streptomyces* JA 3789 with other strains producing tetraene antibiotics and with other related *Streptomyces* species described in the literature indicated similarity of this strain to *Actinomyces rochei* BERGER *et al.*, 1949 emend. GAUSE *et al.*, 1957 (FÜGNER and BRADLER, 1963).

Table 1. Cultural characteristics of *Streptomyces* strain JA 3789

Culture medium	Substrate mycelium		Soluble pigment	Aerial mycelium		Remarks
	Growth	Color		Growth	Color	
Medium I[2] with min. nitrogen (GAUSE *et al.* 1957)	somewhat raised, wrinkled	ivory to pale yellow to dirty yellowish-olive	none	velvety	white turning grey, later brownish-grey	
Medium II[2] with org. nitrogen (GAUSE *et al.* 1957)	raised wrinkled	pale yellow to brownish	none	velvety	white sometimes with greyish tinge	
Sucrose-nitrate agar	thin, flat, transparent	colorless, whitish, pale greyish shade from aerial mycelium	none	powdery to scant	white to pale grey to brownish-grey	
Glucose-asparagin agar	raised	ivory to pale buff	none	velvety	white to pale grey to brownish-grey	
Starch agar	smooth to wrinkled	ivory to pale buff, sometimes faint reddish	none	velvety	white to pale grey to brownish-grey	Hydrolysis : strong
Oatmeal agar	raised	pale buff, sometimes later brownish-grey with olive tinge	none	velvety	spotted light grey, and grey and dark brown grey	
Glucose-yeast-extract agar	abundant wrinkled	light to dark buff to light brown	none	velvety	white becoming faint grey to brown-grey	
Nutrient agar	raised	dirty, cream-colored to faint buff	none	scant	white	
Potato plug	raised, wrinkled	colorless, cream-colored, olive reverse	none	velvety	white becoming spotted light grey and grey	later plug colored dark greyish-olive
DORSET'S egg-medium	raised, wrinkled	yellowish (color of the medium)	none	velvety	white turning faint brownish-grey	Liquefaction : scant
Plain gelatin	flat colony and ring on surface, also dived flocks	colorless, whitish to faint cream-colored	none	velvety	white	Liquefaction : good
Skim milk	ring and heavy surface pellicle	whitish to cream-colored	Peptonized zone : reddish	at the glass broad, powdery border	white	Coagulation : slight or none. Peptonisation : rapid, completed in two weeks
Nitrate broth	surface ring and flaky sediment	whitish to faint cream-colored	none	none		Reduction : weak to medium
Dextrose-nitrate broth	pellicle and flaky sediment	colorless, whitish	none	velvety	white to light grey	Reduction : none or weak
Sucrose medium	growth poor	whitish	none	none or velvety	grey	Inversion : none
Tyrosine medium	little colony on the surface and arborescent growth in the stab	colorless whitish to cream-colored	none	velvety	white to light grey	
Cellulose	no growth					

Table 2. Physiological properties of *Streptomyces* strain JA 3789

Reaction	Results
Melanin formation	none
Nitrate reduction	positive (very weak)
Hydrolysis of starch	positive
Gelatin liquefaction	positive
Milk coagulation	negative or very weak
Milk peptonization	positive

Table 3. Carbon sources utilization by *Streptomyces* strain JA 3789

Growth	Carbon sources
Good growth	D-glucose, levulose, D-mannose, D-galactose, maltose, lactose, trehalose, raffinose, inositol, glycerol, starch, dextrine
Poor growth	mannitol, sodium citrate, sodium succinate
Faint or no growth	D-xylose, D-arabinose, L-rhamnose, L-sorbose, sucrose, dulcitol, sorbitol, salicin, aesculin, inulin, sodium acetae

Later it was possible for us to compare the new organism with authentic strains of the species *Streptomyces rochei* BERGER *et al.*, 1949 (NRRL B-1599, ATCC 10739) and *Streptomyces noursei* HAZEN *et* BROWN, 1950 (ATCC 11455) by cultivation of these strains on standard media.

These investigations indicated that the morphological and physiological characteristics of the strain JA 3789 closely resemble much those of the species *Streptomyces noursei*.

Streptomyces JA 3789 differs from *Streptomyces rochei* ATCC 10739 in the smooth surface of its spores and the shape of its sporophores. The latter forms very long and open spirals or irregular waves. Furthermore their carbon utilization patterns are different.

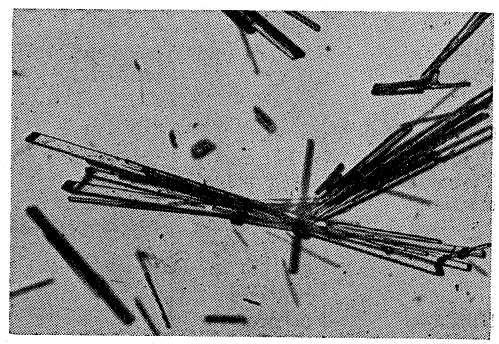
Therefore our strain *Streptomyces* JA 3789 must be considered a variety of *Streptomyces noursei*. In view of differences in the utilization of the carbon sources, raffinose and lactose, and the particular formation of tetramycin it was named *Streptomyces noursei* HAZEN *et* BROWN, 1950, var. *jenensis* nov. var. JA 3789.

Fermentation and Isolation of Tetramycin

Tetramycin was produced readily in shake flasks as well as in fermentors using a culture medium of the following composition: 2% soy bean meal, 2% glucose, 0.5% sodium chloride and 0.3% calcium carbonate, tap water.

In a 600-liter fermentor, 450 liters of medium were prepared and the pH was adjusted to 7.0~7.2. After autoclaving and inoculation of 20 liters of seed culture, fermentation was carried out under the following conditions: temperature 27~29°C, aeration 400 liters/min., agitation 300 r.p.m. Soy bean oil was used as an antifoam agent. The concentration of tetramycin produced in the culture broth reached a maximum at 72~96 hours after inoculation. The antifungal activity of tetramycin was measured by the cup plate method using *Candida albicans* as a test organism. A typical fermentation in the above medium assayed about 150~200 mcg/ml. The antibiotic activity was found mainly in the culture filtrate and partly in the mycelium. Therefore the fermentation broth (400 liters) was separated at pH 4~5 and the antibiotic

Plate 1. Tetramycin crystals.



was extracted from the filtrate with *n*-butanol (130 liters) at neutral pH. The solvent extract was concentrated under reduced pressure to 1/20 of the original volume and a crude product was obtained by precipitating with ether. Purification was further accomplished by using the following method[2]: The crude antibiotic was first converted into an appropriate salt, *e.g.* the triethylaminesulfate double salt. A suspension of the crude antibiotic (150 g) in 1 liter of 20 % methanolic triethylamine sulfate was stirred for 30 minutes and then filtered. The filtrate was evaporated to 500 ml, and 2 liters of acetone were slowly added to precipitate the double salt. The suspension of tetramycin triethylamine sulfate salt was cooled to 0°C overnight and then filtered. The product was washed with acetone - methanol (3 : 1) and dried up *in vacuo* yielding 35 g of a yellow amorphous powder.

The double salt (35 g) was suspended in 500 ml 50 % methanol and dissolved by adjusting the solution of pH 10 with 2.5 N methanolic sodium hydroxide. The solution was filtered and slowly adjusted to neutral pH with 0.5 N methanolic phosphoric acid. Water (150 ml) was then added and after cooling to 0°C, the precipitated antibiotic was collected by filtration and dried *in vacuo* yielding a pale yellow powder (15 g). This crude tetramycin (15 g) was extracted with hot absolute methanol, whereby the pigment-type impurities were removed. The residue was then dissolved in a small amount of pyridine, and after the solution had stood for 24 hours at −5°C the precipitated antibiotic was collected by filtration, washed with ether, and recrystallized from 70 % methanol. About 10 g of colorless needle crystals of tetramycin were obtained (Plate 1).

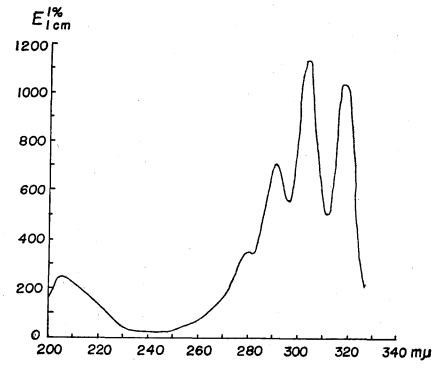
Chemical and Physical Properties of Tetramycin

Tetramycin has no definite melting point; the crystals begin to turn yellow at 150°C and melt at about 260°C with decomposition. The specific rotation of tetramycin is: $[\alpha]$ +89° (*c* 0.5, dimethylformamide), +9.5° (*c* 0.5, pyridine) and +84° (*c* 0.2, 80 % methanol).

Solubility: Tetramycin is readily soluble in dimethylformamide, pyridine, acetic acid, moderately soluble in aqueous alphatic alcohols, aqueous acetone and methylcellosolve, but insoluble in water, benzene, chloroform, and ether.

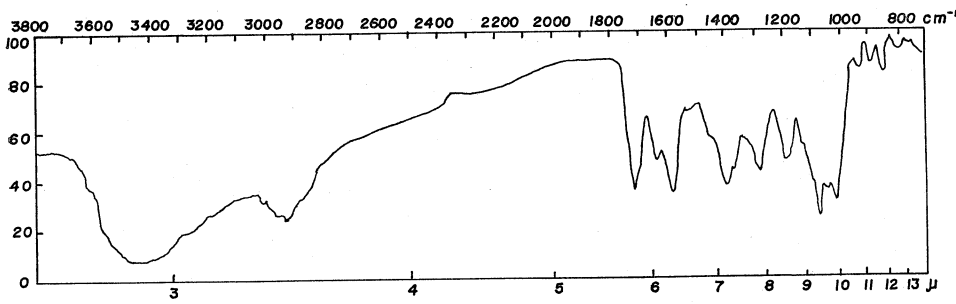
Qualitative chemical tests giving positive results: conc. sulfuric acid (wine red coloration), BAYER, BENEDICT, FEHLING, TOLLENS, ninhydrin, 2,4-dinitrophenylhydrazone, thiosemicarbazone tests. The ferrichloride and MILLON tests are negative. The antibiotic decolorizes a chloroform solution of bromine. Elemental analysis gives the following values: C 58.15, H 7.72, N 1.95 %; $O_{diff.}$ 32.18 %; corresponding to an empirical formula $C_{34}H_{53}O_{14}N$ (molecular weight 699).

Fig. 1. Ultraviolet spectrum of tetramycin in 80 % methanol.



Potentiometric titrations performed in aqueous methanolic solution and in glacial acetic acid revealed the presence of one acid and one basic

Fig. 2. Infrared spectrum of tetramycin (KBr pellet).



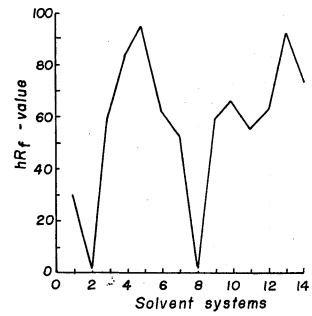
group (equivalent weight 715). Tetramycin is an amphoteric substance and shows pKa-values of 5.1 and 8.6 by titration with tetramethylammonium hydroxide in methylcellosolve[4].

The ultraviolet spectrum of tetramycin in 80 % methanol (Fig. 1) shows fine structure with absorption maxima at 290 nm ($E^{1\%}_{1cm}$ 750), 304 nm ($E^{1\%}_{1cm}$ 1200) and 318 nm ($E^{1\%}_{1cm}$ 1050). The infrared absorption was of the usual type characteristic of polyene antibiotics (Fig. 2). The spectrum shows the presence of either an ester, or lactone group by the band at 1720 cm^{-1}, ionized carboxyl group by that at 1580 cm^{-1}, a polyene system by the band at 1640 cm^{-1}, and hydroxyl-functions by the absorption at 3400 cm^{-1}. The band at 940 cm^{-1} is attributed to a disubstituted trans double bond system.

Quantitative determination of the functional groups gave the following values: methoxyl group, none; acetyl group, none; acetylable groups 9~10.

Catalytic hydrogenation of tetramycin was carried out in glacial acetic acid with ADAM's catalyst under atmospheric pressure. The hydrogen consumption was five moles.

Fig. 3. Summarized papergram of tetramycin.



1 : Water-saturated *n*-butanol (hRf : 30)
2 : 20 % NH_4Cl (hRf : 4)
3 : 3 % NH_4Cl (hRf : 59)
4 : 75 % Aqueous phenole (hRf : 82)
5 : 50 % Aqueous acetone (hRf : 93)
6 : *n*-Butanol - methanol - water - methylorange (40:10:20:1.5g) (hRf : 62)
7 : *n*-Butanol - methanol - water (40 : 10 : 20) (hRf : 54)
8 : Benzene - methanol (80 : 20) (hRf : 0)
9 : Distilled water (hRf : 59)
10 : *n*-Butanol - acetic acid - water (40 : 10 : 50) (hRf : 65)
11 : *n*-Butanol - pyridine - water (60 : 40 : 30) (hRf : 55)
12 : Dimethylformamide - water (10 : 90) (hRf : 62)
13 : Dimethylformamide - water (50 : 50) (hRf : 90)
14 : 70 % Aqueous propanol (hRf : 70)

Hydrolyses of perhydrotetramycin gave a colorless and hygroscopic substance giving a positive reaction with ninhydrin. Rf-values on paper chromatographic analysis using four solvent systems were in agreement with those of mycosamine[5].

The paper chromatographic behavior of tetramycin was investigated using Schleicher & Schüll paper No. 2043 b mgl and *Candida albicans* as test organism in bioautography. Results are given in Fig. 3.

Biological Properties of Tetramycin

Tetramycin is active against yeasts and fungi and inactive against bacteria. The agar dilution method was used to investigate the antifungal spectrum of tetramycin. The spectrum is given as Table 4.

Stability: Tetramycin is stable in the solid state and is also relatively stable in aqueous solutions between pH 7 and 9, it is less stable at pH values below 6 and also unstable in more basic solutions. No loss of activity occurred when a 80 % methanol solution was heated at 60°C for 60~120 minutes in a sealed tube. Tetramycin solutions rapidly lose their activities when exposed to light.

The LD_{50} value of tetramycin in mice is 50 mg/kg determined by a single intravenous administration of gum arabic suspension.

Table 4. Antifungal activity of tetramycin

Strains	Minimal inhibitory concentration (mcg/ml)
Aspergillus niger	15.6
Saccharomyces cerevisiae	15.6
Hansenula anomala SG 908	7.8
Candida albicans SG 916	15.6
Candida albicans SG 942	15.6
Candida krusei SG 937	15.6
Candida tropicalis SG 938	7.8
Trichophytum mentagrophytes var. *interdigitale* SG 955	31.3
Microsporum gypseum SG 958	15.6
Microsporum canis SG 959	15.6

Conclusions

An antifungal antibiotic has been isolated from the culture of *Streptomyces noursei* HAZEN *et* BROWN, 1950, var. *jenensis*, nov. var. JA 3789.

On the basis of its ultraviolet absorption spectrum this antibiotic proved to be a tetraene type substance and has been designated as tetramycin.

Tetramycin differs in its chemical and physical properties as well as paper chromatographic behavior from other known tetraene antibiotics[6~9].

Thus tetramycin must be regarded as a novel antibiotic. The structure studies are under way and will be reported separately.

References

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