ANTIBIOTICS FROM THE GENUS *FUSARIUM*
ENNIATIN B

I. Culture Studies and Antimicrobial Activity

BY M. O. TIRUNARAYANAN AND M. SIRSI

(*Pharmacology Laboratory, Indian Institute of Science, Bangalore* 3)

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SUMMARY

Culture studies on the production of enniatin B have indicated that the optimum conditions are, the use of a nitrate nitrogen source and high carbon : nitrogen ratio. The antibiotic appears mostly in the mycelium under these conditions.

Enniatin B does not appear to be inactivated by moderate concentrations of whole blood, gastric extract and intestinal mucosa, and differences in pH.

Studies on the nature of bacterial inhibition due to enniatin-B indicate that it may interfere with the metabolism of inositol or choline and thereby exert its anti-bacterial effect.

The genus *Fusarium* Link. classed under *Fungi imperfecti*, has been of interest to biologists from very early times. Because of the widespread occurrence of this genus and being causative organism of wilt in a variety of plants of economic importance, attention so far has been restricted to the study of the fungus from points of view of plant pathology and biochemistry.

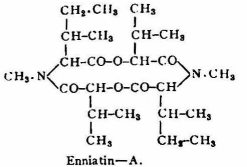
The discovery that this group of fungi is also capable of elaborating antibiotic substances, is of recent origin. The present investigations have been carried out mainly for exploring the possibility of finding *Fusarium* that may elaborate compounds of clinical usefulness in antibiotic therapy, particularly in tuberculosis.

With this object in view, about 130 strains of *Fusarium*, representing about 33 species (as designated by various authors), collected from various parts of the world, have been tested for antibiotic production under different sets of cultural conditions and screened against a variety of organisms representing gram-positive, gram-negative and acid-fast groups.[1]

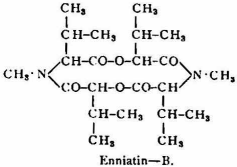
The announcement in 1947 by Gaumann *et al.*,[2] that a number of antibiotics from *Fusaria* have been isolated and found to possess exceptionally high activity against the tubercle bacillus has been received with great interest. One of the antibiotics "enniatin-A" isolated from a strain of *Fusarium orthoceras* App. et var. *enniatinum* [later renamed *Fusarium oxysporum* Schlecht. (Roth, 1954)[3]] has been

shown to possess activity of the order of 1 : 1,000,000 against tubercle bacilli. Enniatin-B has also been found to possess high activity.

Plattner and Nager[a,b,c] and Plattner, Nager and Boller[5] have carried out extensive investigations on the chemistry of these compounds, and have assigned the following structure by chemical degradation studies.



Enniatin—A.



Enniatin—B.

In view of the interest aroused as a result of these investigations, one of the compounds, enniatin-B, has been isolated in this laboratory and its properties studied in a detailed manner. Particular emphasis has been laid to elucidate its action on progressive tuberculous infection in the mouse and also its pharmacological properties.

MATERIALS AND METHODS

The maintenance of the cultures of *Fusarium*, the basic media employed for antibiotic production, and the mode of evaluation of antibiotic activity have been described in detail earlier.[1] In brief, the fusaria were maintained on the potato-dextrose-malt agar of the following composition.

Potato-Dextrose-Malt Agar

Dextrose	30·0 gm.
Malt extract	5·0 gm.
Extract of potatoes representing	400·0 gm.
Agar-agar	20·0 gm.
Distilled water	1000·0 ml.
pH	6·5

The cultures were grown on a modified Czapek-Dox medium of the following composition to study the antibiotic production.

Nitrogen source	1·0 gm.
Potassium dihydrogen phosphate	0·5 ,,
Magnesium sulphate	0·2 ,,
Ferrous sulphate	0·001 ,,
Carbon source	as indicated
Distilled water	100·0 ml.

The agar cup plate method has been used to study the antibiotic production due to cultural variation and the serial dilution technique to assay the antimicrobial activity of *Enniatin* B.

The influence on the growth of the fusaria by alterations in the cultural media was determined by noting the dry weight and the fat content of the mycelium as follows:—

Determination of the Dry Weight of Mycelium.—The mycelia at the end of the incubation period were harvested by filtration over a sintered glass funnel, washed thoroughly with distilled water, partially dried on filter-paper folds and then in a desiccator for 72 hours *in vacuo*. They were then accurately weighed in an analytical balance.

Determination of the Fat Content of Mycelium.—The dry mycelia were powdered and repeatedly extracted with ether and the combined ether extracts evaporated to dryness in a tared dish and weighed. From the difference in the weights of the dish, with and without the ether extract, the total fat of the mycelium and the percentage of fat in the mycelium calculated.

Culture Studies in the Production of Enniatin-B.—A strain of *Fusarium avenaceum* (Fr.) Sacc. (CMI 49894) obtained from the Commonwealth Mycological Institute, Kew, Surrey, has been found to produce maximum activity among the many strains tested.

Effect of Nitrogen.—Antibiotic production as modified by conditions of culture has been studied. In this connection the effect of different types of nitrogen sources, such as nitrate, ammonia, amine, amide and amino-nitrogen, have been examined. The organism grown in the different media for 15 days at room temperature was tested for antibiotic activity using *Micrococus pyogenes* var. *aureus* as the test organism. Nitrogen sources were added at 1% level, with glucose as the carbon source added at 2% level.

The results, presented in Fig. 1, indicate that nitrate nitrogen is having as high an activity as the other sources, such as ammonia and amino nitrogen, while amide nitrogen caused production of only slight activity. Nitrite and amine nitrogen were unable to produce antibiotic activity.

Effect of Carbohydrates.—The influence of a number of carbohydrates on antibiotic production by this strain has been investigated. Among the carbon sources employed, sucrose and glucose presented similar activity. With maltose and fructose, there was no activity, while starch and cellulose showed activity only in the mycelium. Fat determinations have indicated that when starch and cellulose are employed there appeared an increase of mycelial fat with all the activity concentrated within the mycelium, and nothing in the culture fluid. The results of these investigations have been presented in Fig. 2.

Effect of Carbon: Nitrogen Ratio.—The importance of the carbon-nitrogen ratio in antibiotic production in the case of javanicin has been reported earlier,[5]

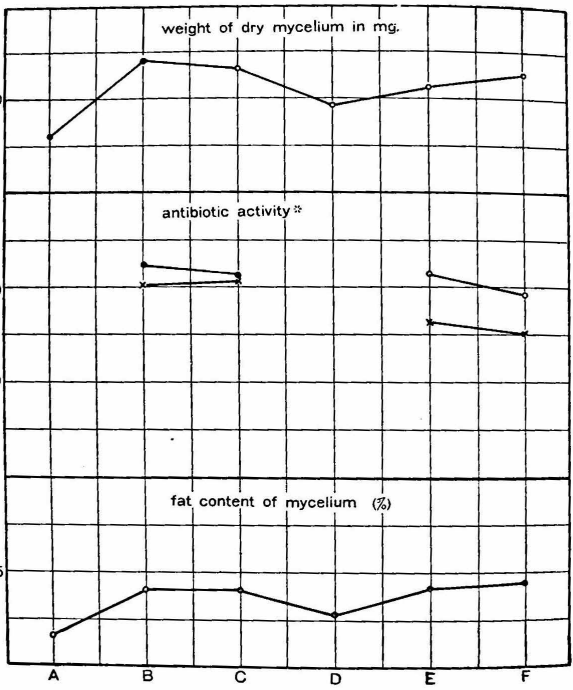


FIG. 1. Effect of Nitrogen Sources on the Growth, Antibiotic and Fat formation by *Fusarium avenaceum* (Fr.) Sacc.

* Millimetres zone of inhibition of *Staphylococcus aureus*.

O———O = culture fluid; ×———× = mycelium

A = nitrite B = nitrate C = ammonia D = amine
E = amino F = amide

During preliminary screening of a large number of *Fusarium* cultures, it has been observed that antibiotic activity produced by most of them appear more in the

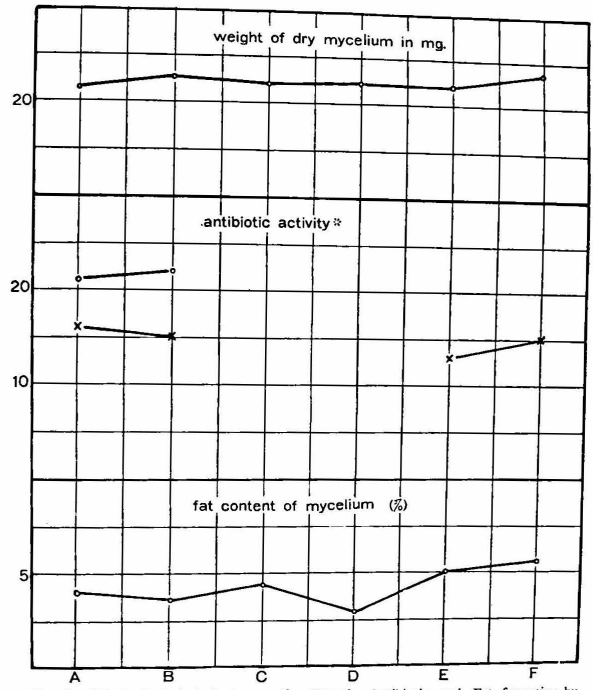


FIG. 2. Effect of Carbohydrates on the Growth, Antibiotic and Fat formation by *Fusarium avenaceum* (Fr.) Sacc.

* Millimetres zone of inhibition of *Staphylococcus aureus*

O———O = culture fluid; ×———× = mycelium

A = glucose B = sucrose C = maltose D = fructose
E = starch F = cellulose

mycelium when the carbon: nitrogen ratio was high. If, however, it is made low, it is found that antibiotic activity appears in the culture fluid, possibly due to cell autolysis.

The effect of different amounts of glucose on antibiotic production by *F. avenaceum* has been studied. In these investigations, sodium nitrate (1%) has been employed as the nitrogen source and incubation was carried out for a period of 17 days. The results, presented in Fig. 3, indicate that beyond a level of 3%

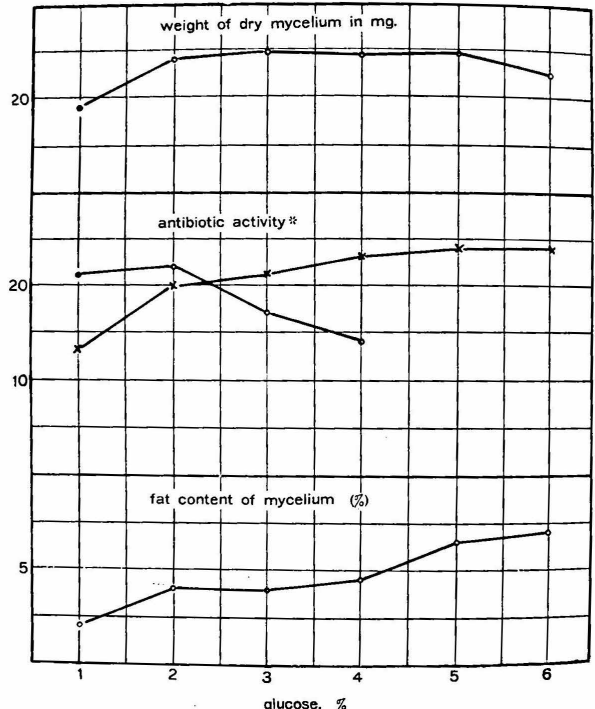


FIG. 3. Effect of Glucose on the Growth, Antibiotic and Fat formation by *Fusarium avenaceum* (Fr.) Sacc.

* Millimetres zone of inhibition of *Staphylococcus aureus*
O——O = culture fluid; ×——× = mycelium.

of glucose, antibiotic activity appeared only in the mycelium, and there was not any detectable amount of activity in the culture fluid.

Effect of pH on Antibiotic Production.—With glucose level at 5% and sodium nitrate (1%), the effect of variations in the initial pH of the medium on the rate of production of antibiotic was studied. The results, presented in Table I, indicate that pH values ranging from 5·5 to 7·0 produce maximum antibiotic activity (15 days growth).

TABLE I

Effect of Initial pH on Antibiotic Production by F. avenaceum (15 *days incubation*)

pH	4·0	4·5	5·5	6·5	7·0	8·0
mm. zone of inhibition of *Micrococcus pyogenes* var. *aureus* by culture fluid (0·1 ml.)	14	20	24	26	25	20

Production and Isolation of Enniatin-B

Fusarium avenaceum (CMI 49894) was grown in 500 ml. culture flasks with flattened base, so as to obtain larger yields of mycelium, on a medium consisting of sodium nitrate (1%) and glucose (5%) and inorganic salts. Following inoculation with a fresh culture of the organism, they were incubated for 20 days at 25–28° C.

The mycelia from the different flasks, at the close of the incubation period, were pooled and dried at a low temperature. The dry mycelium, of about 250 g., was repeatedly extracted with ether and the ether residues combined and evaporated off. The gummy residue which was left behind was redissolved in methanol, and after treatment with norite, filtered and the active substance precipitated by the addition of 15 ml. water to 85 ml. of the methanol extract. By repeated recrystallisations from methanol and petroleum ether, the pure material was obtained. The compound had a sharp melting point of 174° C.

Properties of Enniatins A and B

Plattner, Nager and Boller (1948) have worked out the chemistry of enniatins A and B, and some of the results have been presented in Table II.

Antibiotic Activity of Enniatin-B in vitro

The anti-bacterial activity of enniatin-B has been examined with a large number of gram-positive and gram-negative bacteria. The results reported in Table III indicate that enniatin-B possesses fairly high activity against gram-positive organisms, but does not appear to have any activity against some of the gram-negative organisms.

TABLE II

Some Properties of Enniatins A and B

Compound	m.p.° C.	[a] in $CHCl_3$	Formula	Amino acid after hydrolysis
Enniatin-A ..	121–122	− 91°	$C_{24}H_{42}O_6N_2$	N-methyl-isoleucine
Enniatin-B ..	173–175	−107°	$C_{24}H_{42}O_6N_2$	N-methyl-*l*-valine

TABLE III

Anti-Bacterial Activity of Enniatin-B in vitro

Test organism	Enniatin-B, 1 part in....parts				
	1,000	10,000	100,000	1,000,000	10,000,000
Micrococcus pyogenes var. *aureus* ..	−	−	−	+	+
Streptococcus hemolyticus	−	−	−	+	+
Escherichia coli ..	−	+	+	+	+
Salmonella typhosa ..	−	+	+	+	+
S. paratyphi	−	+	+	+	+
S. schottmuelleri B ..	−	+	+	+	+
Shigella sonne ..	−	+	+	+	+
S. flexneri	+	+	+	+	+
Klebsiella pneumoniæ ..	+	+	+	+	+
Alcaligenes fecalis ..	−	+	+	+	+
Proteus vulgaris OX19 ..	+	+	+	+	+
P. vulgaris OXK ..	−	+	+	+	+
P. vulgaris OX2 ..	+	+	+	+	+
Vibrio choleræ-ogawa ..	−	+	+	+	+
V. choleræ-inaba ..	−	+	+	+	+

− = Inhibition
+ = Growth

Studies on the Nature of Bacterial Inhibition by Enniatin-B

In order to investigate whether enniatin-B inhibits the growth of bacteria by blocking the functioning of any metabolite, a number of vitamins, amino acids and miscellaneous compounds, listed in Table IV, were tested for their effects on the anti-bacterial activity of the compound.

TABLE IV

Substances Tested for Effects on the Activity of Enniatin-B against Staphylococcus aureus

Test substance	Concentration (M)	Test substance	Concentration (M)
Vitamins		*Amino Acids*	
Ascorbic acid ..	0·01	Glutamic acid ..	0·005
Thiamine ..	0·01	Aspartic acid ..	0·005
Riboflavin ..	100 γ/ml.	Tryptophane ..	0·01
Nicotinic acid ..	100 γ/ml.	Glycine	0·1
Pyridoxine ..	100 γ/ml.	Methionine ..	0·001
Inositol	500 γ/ml.	Cysteine	0·01
Choline	200 γ/ml.	Threonine ..	0·001
p-Aminobenzoic acid	100 γ/ml.	Serine	0·001
Folic acid ..	10 γ/ml.	Histidine ..	0·005
Menadione (K-3) ..	25 γ/ml.	Arginine ..	0·005
Ca. pantothenate ..	100 γ/ml.	Leucine ..	0·001
Biotin	1 γ/ml.	Valine	0·001
Cations (as chlorides)		*Anions* (as sodium salts)	
Na	0·01	Cl	0·01
K	0·01	CO_3	0·001
Mg	0·0001		
Mn	0·001	SO_4	0·0001
Ca	0·0001		
Fe (as sulphate) ..	0·00001	PO_4	0·002
		Miscellaneous	
		Glutathione ..	0·001
		Semi-carbazide ..	0·000001

Among the compounds tested only inositol and choline showed inhibitory effects on the activity of enniatin-B. The inhibition obtained with inositol was nearly 80% while that with choline was only 50%. The possibility that enniatin-B may exert its anti-bacterial action by competing with either inositol or choline as a result of similarity in chemical structure seems promising enough, and would form the basis for further investigations. The increased activity of enniatin-B with menadione has been found to be due to an inhibitory effect of the latter compound on the growth of the organism.

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