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86. Tatsuzo Fujii : Biochemical Studies on Pathogenic Fungi. VIII.* The Effect of an Antibiotic, Trichomycin, on the Respiration and Phosphorus Metabolism of *Trichophyton gypseum*.

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An antibiotic, Trichomycin, discovered by Hosoya, is reported to be effective against several species of pathogenic fungi including dermatophytes and also against some kinds of protozoa.[1] The present author already investigated the action of several antibiotics on the growth of a dermatophyte, *Trichophyton gypseum*, and found that only Trichomycin inhibited the growth in concentrations over 200 γ/cc., while others, penicillin, streptomycin, and tetracycline antibiotics, were without effect.[2]

As an approach to the elucidation of the mode of its antimycotic action, effect of Trichomycin on various phases of metabolism of *T. gypseum* was investigated by the author. The results so far obtained revealed the remarkable effect of this antibiotic upon the phosphorus metabolism as well as on the respiration of this dermatophyte, and these results are reported herein.

Effect of Trichomycin in a series of concentrations from 0.01 to 10,000 γ/cc. on glucose respiration of *T. gypseum* was determined and percentage of the resulting maximum increase or decrease in the respiratory rate against the normal level was calculated (Fig. 1). In a range of about 0.1 to 100 γ/cc., definite stimulative action on

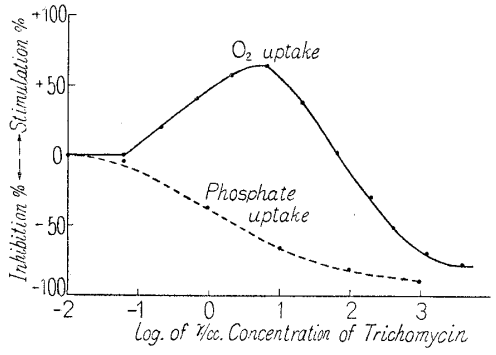

Fig. 1.

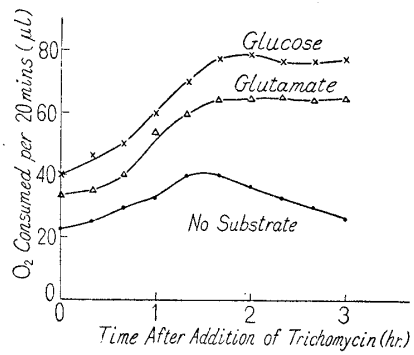

Fig. 2.

oxygen consumption was observed, while over this concentration inhibitory effect appeared which increased gradually with increasing concentration. Such an effect did not change significantly at pH between 5.5 and 8.5. The change in the respiratory rate of the cells after the addition of Trichomycin in 10 γ/cc. is pictured in Fig. 2. The rate gradually increased upon its addition until reaching the maximum about 1.5 hours later. When a suitable energy source such as glucose or glutamate was present, the cells maintained the high rate, while the respiratory rate decreased gradually in its absence.

Such a marked stimulative effect of Trichomycin in a very minute amount on respiratory oxygen uptake of this fungus, which apparently was not due to oxidative breakdown of the antibiotic molecule itself by the fungal metabolism, seems to be

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1) S. Hosoya : Chemotherapy (Japan), **2**, 1(1954).
2) T. Fujii : Unpublished data.

somewhat unusual at first sight, but several compounds including natural products are known to exert such an effect on some organisms in a minute concentration. Among them are noted one group of the so-called "uncouplers" against oxidative phosphorylation system,[3,4] including dinitrophenols,[5] tetracyclines,[6] and ipomeamarone,[7] etc., which specifically inhibit phosphorylating activity by uncoupling it from the energy-supplying, oxidative mechanism while not injuring, or sometimes even stimulating, the latter activity.[4] Such a fact made the author attempt the examination of the effect of Trichomycin on the phosphorus metabolism of *T. gypseum*. How this antibiotic affected the phosphate uptake of this fungus cells from the surrounding medium was studied with the aid of radioactive phosphate added to the medium.

The cell suspension of the fungus obtained by shake culture as reported previously[8] was incubated with 40 μmole glucose, 13.3 μmole phosphate, and 8.2 μc. of ^{32}P-labeled phosphate (specific activity, 0.25 mg./mc.) in a Warburg flask at 30°. Trichomycin was added to give the final concentration of 30 γ/cc., and as the control run, water instead of Trichomycin solution was added. After 3 hours' incubation, trichloroacetic acid was added to stop metabolic reactions. The fungal cells, after fractionating into acid-soluble and -insoluble parts, and the surrounding medium solution, were analyzed for their ordinary and radioactive phosphorus contents. In order to detect the amount of ^{32}P initially present as well as the amount adhered on the cell surface, blank was run to which trichloroacetic acid was introduced just after the addition of ^{32}P and other components to the cell suspension.

Table I.

	Trichomycin added (γ/cc.)	Incubation time (hr.)	Content in the surrounding medium	Content in acid-soluble fraction of the cell
Determination of P (γ)	0	0		10.2 (51.0)
	0	3	*	13.0 (65.0)
	30	3		11.8 (59.0)
Radioactivity of ^{32}P (counts/min.)	0	0	4,130 (123,900)	466 (1,398)
	0	3	2,841 (85,230)	2,875 (8,625)
	30	3	3,898 (116,900)	744 (2,322)

The value represents the amount per aliquot, and that in parentheses is the amount calculated for the whole fraction.

* Not determined, because the yellow color of Trichomycin solution disturbed the colorimetric determination of phosphate.

The amount of phosphorus and radioactivity in aliquot of each fraction are indicated in Table I. The value calculated per whole fraction is also cited in parentheses in the Table. Of the ^{32}P initially present in the medium, the amount that remained after 3 hours' incubation with the respiring cells was 94% in the presence of Trichomycin as compared to about 70% in its absence. Thus, as a result of the action of this antibiotic, the amount of phosphate consumed from the surrounding medium by uptake by the cells was reduced to one-fifth of the normal amount (from 30% to 6% of the initial dose).

Because some radioactivity was detected in the acid-soluble fraction of the cell even in the blank test, possibly due to its nonspecific adsorption on the cell surface, this amount of ^{32}P was subtracted from the experimental value. Such corrected amount of radioactivity in the fraction was divided by the amount of ordinary phos-

3) T. M. Brody : Pharmacol. Rev., **7**, 335(1955).
4) I. Uritani, T. Akazawa : Kagaku no Ryoiki (Japan), Suppl. No. 20, 200(1955).
5) W. F. Loomis, F. Lipmann : J. Biol. Chem., **173**, 807(1948).
6) W. F. Loomis : Science, **111**, 474(1950).
7) I. Uritani, T. Akazawa, M. Uritani : Nature, **174**, 1060(1954).
8) T. Fujii : This Bulletin, **5**, 506(1957).

phorus present there, thus specific activity was calculated. As shown in Table II, it is apparent that the specific activity of the acid-soluble phosphorus of the Trichomycin-treated cell was about 14% of that of untreated cell. This result may be indicative of the fact that the activity of phosphate uptake (probably due to oxidative phosphorylation) of this dermatophyte was markedly inhibited by this antibiotic.

Table II.

Trichomycin added (γ/cc.)	^{32}P specific activity* of acid-soluble fraction of the cell
0	115.2
30	15.7

* Radioactivity : count/min./γ P.

Effect of Trichomycin in a series of concentrations upon phosphate uptake by the fungus cells was examined by determining the decreased rate of disappearance of radioactive phosphate from the surrounding medium and percentage inhibition was plotted against logarithm of concentration (Fig. 1). The inhibitory effect was observed from as low as 0.1 γ/cc. where this antibiotic began to exert stimulating action upon respiration of this fungus.

These results clearly show that the antibiotic Trichomycin, in concentration which does not inhibit but rather stimulate the respiratory rate of *T. gypseum*, specifically inhibits the phosphate uptake of the fungus cells from the surrounding medium. Such an action of Trichomycin upon the fungal metabolism may be taken as at least one cause of the antimycotic activity of this antibiotic against this dermatophyte. The direct demonstration of its action against the oxidative phosphorylation system of this fungus will be made in the near future with mitochondrial preparation isolated from the cells.

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Experimental

Trichomycin Preparation Tested—Partially purified Na salt of Trichomycin with the activity of 8,000 trichomonas unit[1] per mg. was used, which was supplied by the Fujisawa Pharm. Ind., Ltd., by the permission of Dr. Hosoya, the discoverer of this antibiotic. It was easily soluble in water and gave yellow colored solution.

The Organism Employed—The same normal strain of *Trichophyton gypseum* as used in the previous study[8] was grown in 2% glucose-peptone broth by shake culture for 4 days at 28°. The fungus pellets thus harvested were suspended in isotonic KCl solution after being washed with water. The suspension usually contained about 10 mg. cells in dry weight per cc. The details were described already[8].

Measurement of the Respiratory Oxygen Uptake of the Cells—The rate of O_2 uptake was measured by the conventional Warburg manometry at 30°. Usually one flask contained 1.0 cc. of cell suspension, 0.8 cc. of *M*/15 phosphate buffer of pH 7, and 0.2 cc. of *M*/4 glucose solution. 0.5 cc. of Trichomycin solution of appropriate concentration was added from the side arm. The rate of uptake was measured in 20-min. intervals, and percentage inhibition or stimulation upon the rate caused by this antibiotic was calculated as described previously.[8]

Fractionation and Determination of Phosphorus Compounds—After adding CCl_3-COOH to the reaction mixture to 5%, the medium solution was centrifuged off, and the cells were washed twice with 3 cc. of 5% CCl_3-COOH. The combined supernatant and the washings were made to 15 cc. with water.

The cells were homogenized twice with 2-cc. portions of 10% CCl_3-COOH and with 2 cc. of its 5% solution. After the residue was washed with 3 cc. of its 5% solution, all supernatants were combined. This acid-soluble fraction of the cells was digested with $HClO_4$ and H_2O_2 on a sand bath and the digest was made to 15 cc.

From the above two fractions with the total volume of 15 cc., 0.5- and 3.0-cc. aliquots were taken in order to determine phosphate P originating from P compounds in each fraction, and 0.5 and 5.0 cc. each to determine the radioactivity of ^{32}P-labeled phosphate. Inorganic phosphate was determined colorimetrically using the method of Fiske and Subbarrow.[9] Radioactive phosphate was precipitated together with carrier phosphate (1 cc. of 0.1 *M* solution) by Mg mixture and NH_4OH, and the precipitate was filtered, washed with MeOH, transferred to an aluminum dish, and dried *in vacuo*. Radioactivity of the samples thus prepared was measured by the Geiger-Müller counter. The data were corrected for the background.

Summary

The action of Trichomycin on the respiration and phosphorus metabolism of *Trichophyton gypseum* was examined. This antibiotic stimulated the respiratory rate of this fungus in a range of 0.1~100 γ/cc., while it inhibited the rate markedly over 100 γ/cc.

The inhibitory action of Trichomycin upon the incorporation of phosphate into the cells from the surrounding medium was demonstrated with the aid of ^{32}P-labeled phosphate. Such an effect appeared from 0.1 γ/cc.

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9) C. H. Fiske, Y. Subbarow : J. Biol. Chem., **66**, 375(1925).