

Basal Medium	{	MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.1 g.
		M/15 Phosphate buffer (pH 7.0)	100 cc.
		Trace element solution*	0.5 cc.
		Distilled water,	to make 1000 cc.

\* Containing H<sub>3</sub>BO<sub>3</sub>, CuSO<sub>4</sub>, MnSO<sub>4</sub>, ZnSO<sub>4</sub>, and iron alum.

**Measurement of Growth on Nitrogen Source**—To the basal medium, respective nitrogen source was added to give desired concentration and glucose as carbon source to 2%. Inoculation was made in a 100-cc. Erlenmeyer flask containing 30 cc. of such medium. Other details were the same as above.

### Summary

Carbohydrate utilization and nitrogen requirement of *Trichophyton gypsum* were studied and differences in the nutritional physiology between the normal and pleomorphic forms of this fungus were demonstrated. The pleomorphic form showed better growth and more efficient assimilation on monosaccharides than the normal form. Di- or polysaccharides generally failed to support appreciable growth of either form. The pleomorphic form grew far more readily on single amino acid than the normal form, and reproduced even on ammonium nitrogen to some extent which the normal form could not utilize at all.

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### 85. Tatsuzo Fujii : Biochemical Studies on Pathogenic Fungi. VII.\* The Effect of Synthetic Fungicides and Fatty Acids on the Respiration of *Trichophyton gypsum*.

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As a means of evaluating the *in vitro* activity of antibacterial compounds, attempts have been made by several workers to take the degree of their inhibitory action upon the respiration of some representative species.<sup>1-3)</sup> This method was applied to dermatophytes by Nickerson.<sup>4,5)</sup> According to him, such "metabolic assay method" is more advantageous than the prevailing growth- or germination-inhibition test, because by the former method the direct effect of the compound on respiration of the preformed "adult" fungus mycelium, which is apparently a critical index of the fungal metabolism, can be determined quantitatively.

Applying the findings obtained by the previous study<sup>6)</sup> on the respiration of a dermatophyte, *Trichophyton gypsum*, inhibitory action of several antimycotic compounds on the rate of glucose respiration of this fungus was examined here. In dealing with this organism, particular precaution was taken as to the culture forms *in vitro*. Owing to the significant differences in some physiological behavior as well as in morphology

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between the normal (sporulating) and pleomorphic (sterile) forms of the same species,<sup>7)</sup> the effect of the compound to be tested on the respiration of *T. gypseum* was always determined with each of these two forms separately in order to compare their sensitivity to the compound.

Considering the fact that higher fatty acids are of particular interest concerning the prevention of dermatophyte infections, not only due to their powerful antimycotic activity<sup>8)</sup> but also due to their rich occurrence in adult hair fat, which was considered to be one of possible natural defence mechanisms against the infection,<sup>9)</sup> their effect on the respiration of *T. gypseum* was investigated in detail and described in the latter half of this report.

Aqueous solution of the compound under examination was added to the respiring cell suspension of this fungus in the presence of glucose and phosphate buffer (pH 7.0) in a Warburg flask, the resulting change in the rate of oxygen uptake was measured, and the degree of inhibition or stimulation (in percentage against the normal rate) was obtained as described in the experimental part. Such method guaranteed the reproducibility of the data sufficient for the practical application as revealed from the result with 0.001*M* undecylenic acid as an example, where with ten samples, each percentage inhibition agreed within 2.1% from the average of 86.5.

### I. The Effect of Fungicides

The effect of an organic thiomeric fungicide, butyl butylmercurithiosalicylate, a cationic detergent, lauryltriphenylphosphonium bromide, and unsaturated fatty acid, undecylenic acid, in a series of concentrations on the rate of oxygen uptake of this fungus are shown in Fig. 1, where the percentage inhibition is plotted against loga-

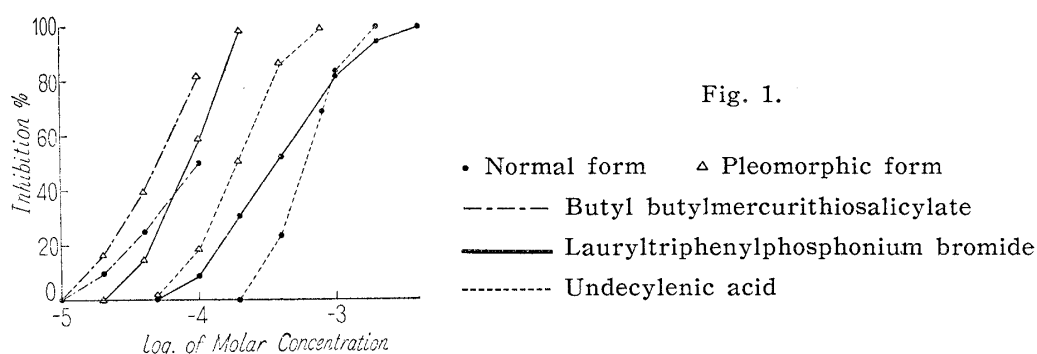


Fig. 1.

TABLE I.

Compounds tested	Molar concn. causing 50% inhibition of respiration		Min. molar concn. preventing growth	
	N-form <sup>a)</sup>	P-form <sup>a)</sup>	N-form	P-form
Butyl butylmercurithiosalicylate	$1 \times 10^{-4}$ (1 : 20,000) <sup>b)</sup>	$5 \times 10^{-5}$ (1 : 40,000)	$5 \times 10^{-6}$ (1 : 400,000)	$2.5 \times 10^{-6}$ (1 : 800,000)
Octylmercurithiobutanol	$1 \times 10^{-4}$ (1 : 25,000)	$6.5 \times 10^{-5}$ (1 : 38,000)	$5 \times 10^{-5}$ (1 : 50,000)	$2 \times 10^{-5}$ (1 : 125,000)
Lauryltriphenylphosphonium bromide	$4 \times 10^{-4}$ (1 : 5,000)	$8 \times 10^{-5}$ (1 : 25,000)	$2 \times 10^{-5}$ (1 : 100,000)	$1 \times 10^{-5}$ (1 : 200,000)
Lauryldimethylbenzylammonium chloride	$5 \times 10^{-4}$ (1 : 6,000)	$2 \times 10^{-4}$ (1 : 15,000)	$5 \times 10^{-5}$ (1 : 60,000)	$1 \times 10^{-5}$ (1 : 300,000)
Undecylenic acid	$6 \times 10^{-4}$ (1 : 9,100)	$2 \times 10^{-4}$ (1 : 27,000)	$3 \times 10^{-4}$ (1 : 18,000)	$1 \times 10^{-4}$ (1 : 54,000)

a) N-form=normal form, and P-form=pleomorphic form, of *Trichophyton gypseum*.

b) Figures in parentheses indicate approximate value of dilution corresponding to the molar concentration.

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rithm of molar concentration of the agent. Another organic mercurial, octylmercurithiobutanol, and another invert soap, lauryldimethylbenzylammonium chloride, showed similar effect but in a slightly higher concentration than the respective analogs.

Concentration of each compound to suppress the respiratory rate of *T. gypseum* to 50% of the normal level was obtained from the above curves. Furthermore, in comparison, the minimal concentration of each agent required for preventing the *in vitro* fungal growth on glucose-peptone medium was determined by the ordinary method. These results are summarized in Table I. Generally, close relationship was noted between these two kinds of effect. The compound with the highest inhibitory action on the fungus respiration was butyl butylmercurithiosalicylate, which was followed by octylmercurithiobutanol, lauryltriphenylphosphonium bromide, lauryldimethylbenzylammonium chloride, and undecylenic acid. Such order agreed fairly well with that concluded from the growth-inhibition power of the agent.

It is interesting to note that all the compounds tested brought about one-half inhibition on the respiration of the pleomorphic form in 1/2 to 1/5 concentration of that causing half-inhibition on the normal form of *T. gypseum*. It was also true for the growth-inhibiting activity of these compounds. This fact may indicate about two- to five-fold increase in sensitivity of the fungus cells to the antimycotic action of these agents as a result of pleomorphic degeneration. From such findings, upon testing the antimycotic activity of a compound on dermatophyte which easily shows such transformation on *in vitro* culture, the importance of selecting the exact normal form of this organism would be understood in obtaining reproducible results.

## II. The Effect of Fatty Acids

As a more detailed examination on the action of fatty acids in general on the respiration of dermatophyte, effect of environmental pH and of their structures on the action is demonstrated below. Furthermore, such effect on dermatophyte was compared with that against the respiration of some common bacteria.

The effect of undecylenic acid was tested at various pH values. In  $3 \times 10^{-4} M$ , this acid was found to inhibit the oxygen uptake of *T. gypseum* below pH 6.5 and increased the effect with increasing acidity, while above this pH it rather stimulated the uptake markedly. Therefore, the action of this acid in a series of concentration was measured at an acid (pH 5.5) and alkaline pH (8.0). As is clear from Fig. 2, this acid was

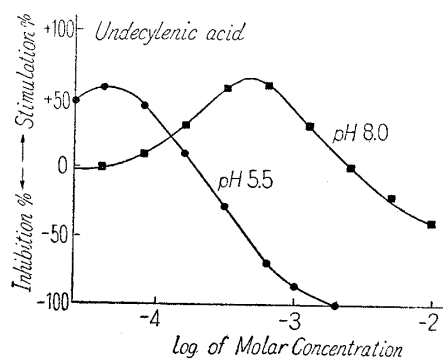


Fig. 2.

found to stimulate the respiratory rate in either pH in a certain range of concentrations below that causing respiratory inhibition. Furthermore, it was proved that the inhibitory effect changed appreciably with environmental pH, the effect being far more stronger in acid than in alkaline medium. It is likely that the undissociated molecule of the acid plays a part here.

Similar experiments were carried out with each member of even-numbered, saturated fatty acids, from  $C_6$  to  $C_{16}$  (Table II). Every acid showed a definite stimulating effect. Such increased oxygen consumption of the fungus cells caused by the



of these bacteria. These facts may reveal the very sensitive nature of the mold cells under study to the action of fatty acids in general as compared with these bacteria.

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### Experimental

**Compounds Tested**—The organic mercurial fungicides were donated by the Kaken Chemicals Co., the cationic detergents from the Kowa Chemicals Co., and undecylenic acid from the Maruishi Pharm. Co. Commercial samples of fatty acids were purified by distillation *in vacuo* before use. Though every compound was tested in aqueous solution, the organic mercurials with low solubility were first dissolved in EtOH, which was then diluted with distilled water. Here, the EtOH content was adjusted not to exceed 1% under which concentration it did not affect the respiration or growth of this fungus.

**Organisms Employed**—*Trichophyton gypseum* O.P.H.-A 801, originally isolated by Dr. S. Ikeda of Osaka Prefectural Hospital, and a pleomorphic strain isolated from it by the present author were used. The detailed description on these strains were made in the preceding paper.<sup>7)</sup>

The dermatophyte was grown in a 500-cc. shake-culture flask containing 100 cc. of glucose-peptone medium of the following composition with continuous shaking (100 cycles/min., in 7.5 cm. path) at 28° for 4 days. The fungus pellets harvested were thoroughly washed with water and suspended in isotonic KCl solution. The suspension usually contained about 10 mg. of cells in dry weight per cc.

{	Glucose	20.0 g.
	Peptone (Polypeptone Takeda)	10.0 g.
	MgSO <sub>4</sub> ·7 H <sub>2</sub> O	0.1 g.
	M/15 Phosphate buffer (pH 7.0)	100 cc.
	Trace element solution*	0.5 cc.
	Distilled water,	to make 1000 cc.

\* Containing H<sub>3</sub>BO<sub>3</sub>, CuSO<sub>4</sub>, MnSO<sub>4</sub>, ZnSO<sub>4</sub>, and iron alum.

The bacterial strains used in the comparative experiments were *Staphylococcus aureus* 209 P and *Escherichia coli* B. They were grown on ordinary agar plate at 37° for 18 hrs., and cells obtained were washed with saline and suspended in isotonic KCl solution, so that 1 cc. of this suspension contained about 1 mg. of cells in dry weight.

**The Respiration Inhibition Test**—Usually a Warburg flask contained 1.0 cc. of cell suspension, 0.8 cc. of M/15 phosphate buffer, and 0.2 cc. of M/4 glucose solution in the main chamber, 0.5 cc. of the test solution in the side arm, and 0.2 cc. of 15% KOH in center well. After equilibration at 30°, the test solution or water in control run, was added to the main chamber and rate of O<sub>2</sub> uptake per 20 mins. was measured for 60 mins. The amount of O<sub>2</sub> consumed during the last 20-min. interval was reduced from that of the control run, and percentage of the difference against the control value was calculated, indicating the percentage inhibition or stimulation on respiration caused by the agent being tested.

**The Growth Inhibition Test**—Aqueous solution of the compound was added to test tubes containing 5 cc. of glucose-peptone medium to make double or triple dilution series and to these, inoculation was made from stock culture on glucose-peptone agar. After 10 days' incubation at 28°, the lowest concentration of the compound which completely prevented the growth was observed.

### Summary

The effect of synthetic fungicides and fatty acids on the respiration of normal and pleomorphic forms of *Trichophyton gypseum* was examined manometrically.

Organic thiomercuric fungicides, cationic detergents, and undecylenic acid markedly inhibited the respiration in as low as 10<sup>-3</sup> to 10<sup>-5</sup> molar concentration. Close relationship was observed between their respiration- and growth-inhibiting effects. The pleomorphic form was much more sensitive to the action of these compounds than the normal form of this fungus.

Every saturated fatty acid of even-numbered series from C<sub>2</sub> to C<sub>16</sub> stimulated the respiratory rate of this fungus in a certain range of concentrations, while higher members among them above C<sub>8</sub> inhibited it in relatively higher concentration. The inhibitory action was stronger in the undissociated state than as anion of the same acid, and was the maximum with the C<sub>12</sub> acid. This dermatophyte was more sensitive to the action of these acids than bacterial species examined.

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