Comparative Wax Esterase Activity of Various Microorganisms.* (24484)

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Since Miyoshy's(10) demonstration that paraffin could be utilized as sole carbon source by a penicillium species, a number of reports (6,12,13) have confirmed and extended this observation. Microorganisms and higher organisms capable of breaking down beeswax (2,4,5,6,14) and tubercle wax(3,4,6,8,9,11) have been described. In view of the usefulness of wax dissolving enzymes, presumably esterases, in metabolic studies of tubercle bacilli, more exact information on occurrence and characteristics of such enzymes appeared desirable. The data here presented show that wax esterases, active against beeswax, tubercle wax and tributyrin can be demonstrated in cell free extracts from a variety of microorganisms even though esterase activity of the intact cells may be negligible.

Materials and methods. Organisms for study were *Micrococcus ureae* (ATCC 408), *Aspergillus flavus* (ATCC 9643), *Bacillus macerans* (ATCC 290), *Micrococcus cerolyticus* (ATCC), *Candida albicans* (isolated from clinical material from Passavant Hospital, Chicago), a penicillium species and a Gram-negative rod isolated from commercial beeswax),§ and GM1 (Gram positive rod isolated from intestinal tract of wax moth (*Galleria mellonella*).‖ Stock cultures were maintained on tryticase soy agar slants. *Aspergillus flavus* and the penicillium species were grown in synthetic medium containing 1 g NH_4NO_3, 1 g KH_2PO_4, 20 mg $MgSO_4$, 0.1 g NaCl, 0.1 g $CaCl_2$, 3 mg $FeCl_3$, 1 mg biotin, 10 g dextrose, 1000 ml double distilled water. *Candida albicans,* GM1, the Gram negative rod, *Bacillus macerans* were grown in synthetic medium consisting of 6 g $(NH_4)_2HPO_4$, 0.2 g KH_2PO_4, 40 mg $MgCl_2$, 1 mg biotin, 10 g dextrose, 1000 ml of double distilled water. *Micrococcus ureae* and *Micrococcus cerolyticus* were grown in tryticase soy broth (BBL). To obtain sufficient growth for manometric studies a shallow layer of medium (300 ml) in a 5 liter diphtheria toxin bottle was inoculated with the growth from a slant suspended in 10 ml of culture medium. Cells in the logarithmic phase of growth were harvested by centrifugation and washed 3 times with distilled water. The cells were resuspended in distilled water and adjusted to a standard optical density which corresponded to a nitrogen content of 0.07-0.18 mg/ml depending on strain or microorganism. Nitrogen was determined by the method of Ma and Zuazaga (7). Cell extracts were prepared as follows: Cells in logarithmic growth were harvested, washed 3 times with distilled water, and resuspended in 15 ml of distilled water. Cell suspensions (containing 5-10 g wet wt) of all microorganisms except *Aspergillus flavus* and the penicillium species were subjected to sonic oscillation for 1 hour in a Raytheon 10 KC, 250 watt oscillator. To facilitate breakage of *Candida albicans* the washed cells were lyophilized and 5 times their weight of acid washed glass beads (75 μ) were added. This was then resuspended in 15 ml of distilled water and subjected to sonic oscillation for 1 hour. Suspensions of *Aspergillus flavus* and the penicillium species were ground by hand for 15 minutes in a chilled mortar containing sufficient alumina 303 to produce a thick paste. Treated cell suspensions were then centrifuged at 2900 x g for 15 minutes at 5°C. The crude extracts were maintained at 5°C in an ice bath and saturated with a mixture of 95% N_2 and 5% CO_2 by bubbling in the gas mixture slowly for 5 minutes. One ml of these extracts was used per Warburg vessel and contained from 0.4 to 1.4 mg N_2/ml. Oxygen uptake was determined by conventional Warburg technics at 37°C. Esterase activity was deter-

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‖ Kindly submitted by Mr. R. M. Lycette, Villa Park, Ill.

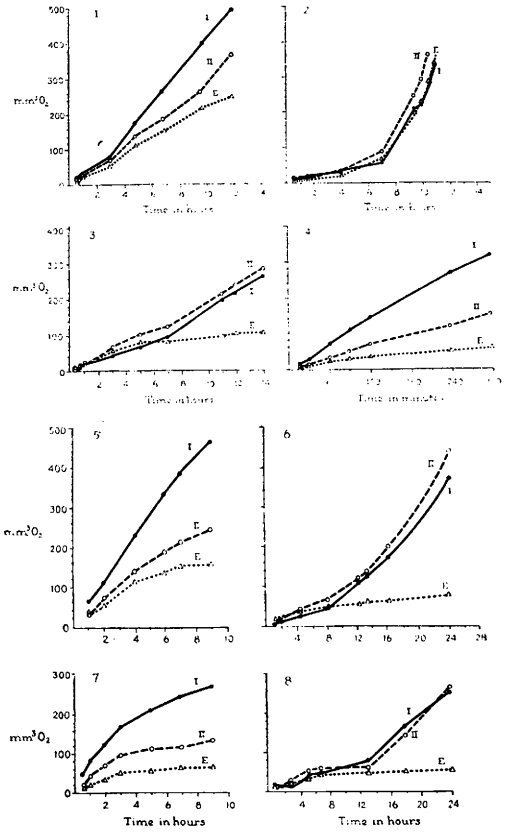

FIG. 1-8.

1, *Aspergillus flavus;* 2. Penicillium species; 3, GM1; 4, Gram negative rod; 5, *Micrococcus ureae*; 6, *Bacillus macerans;* 7, *Candida albicans*; 8, *Micrococcus cerolyticus.*

Contents of Warburg flask: 1 ml of 0.1 M potassium phosphate buffer pH 7.4, substrate, 1 ml of cell suspension containing the following amounts of nitrogen: 0.11 mg *(Aspergillus flavus)*, 0.18 mg (Penicillium species), 0.08 mg (GM1), 0.11 mg (Gram negative rod), 0.1 mg *(Micrococcus ureae)*, 0.07 mg *(Bacillus macerans)*, 0.1 mg *(Candida albicans)*, 0.08 mg *(Micrococcus cerolyticus)*. Center well contained 0.2 ml of 40% KOH. Temp. 37°C. I, Myricyl palmitate; II, TB wax; E, endogenous.

mined by measuring liberation of CO_2 from 0.445 M $NaHCO_3$, pH 7.9, in an atmosphere consisting of 95% N_2 and 5% CO_2, over a period of 24 hours. Myricyl palmitate (Bios), 25 mg/flask; tubercle wax (prepared by the method of Anderson, 1), 25 mg/flask; and tributyrin (Calif. Fn. for Biochem. Research), 0.2 ml/flask were used as substrates. Before use, the first 2 substrates were ground to a fine powder in a mortar immersed in dry ice. The 3 substrates appeared to contain only traces of reducing sugar (Benedict's reagent) and gave negative biuret and ninhydrin reactions.

Results. Oxygen consumption by washed cells in absence (endogenous) and in presence of either myricyl palmitate or tubercle wax are shown in Fig. 1-8. The microorganisms tested differed in both rate and extent to which they attacked the substrates. Higher endogenous values were observed when cells were washed with potassium phosphate buffer than with distilled water.

To determine whether initial metabolic activity of intact cells was due to release of enzyme into the environment, cell free culture broths as well as lyophilized cell free culture broths were tested. All produced negative results.

Only *Candida albicans* showed esterase activity with the substrates tested producing a $Q^N_{CO_2}$ of 92 with myricyl palmitate and 25 with tubercle wax. Negative results obtained with the other microorganisms tested were not due to lack of esterase since extracts of all microorganisms produced positive esterase activity against the wax substrates and tributyrin (Table I). No esterase activity of cell free culture broth could be demonstrated.

Discussion. Increase in oxygen consumption of washed suspensions of 8 cultures in presence of myricyl palmitate and tubercle wax indicated that these microorganisms possessed lipolytic enzyme systems capable of deriving energy from wax esters and it appears likely that some of these enzymes, presumably esterases, are of rather common occurrence.

TABLE I. Esterase Activity of Crude Cell Extracts of Various Microorganisms.

	$Q^N_{CO_2}$		
Extract source	Tributyrin	Tubercle wax	Myricyl palmitate
C. albicans	22	22	27
M. cerolyticus	71	10	27
M. ureae	17	12	68
B. macerans	1528	16	21
A. flavus	396	21	35
Penicillium sp.	495	3	13
Gram neg. rod	33	11	19
GM1	58	20	36

Each Warburg vessel contained 1.2 ml of bicarbonate buffer pH 7.9 (1 ml in case of tributyrin), substrate and 1 ml of extract.

Pronounced lag phase in utilization of substrates by GM1, *Bacillus macerans*, and *Micrococcus cerolyticus* might suggest that adaptation occurs during this period. However, the finding of esterase activity in cell free extracts of unadapted cells speaks against this possibility. In view of the apparent absence of wax esterase in the growth medium it also seems unlikely that the lag might have been caused by the time required for autolysis to liberate esterase. The results are compatible, however, with the view that saturation with endogenous substrate of the sites of esterase or other enzymes further along the chain of reactions might have been the cause of the observed lag.

Summary. 1) Various microorganisms were studied for ability to metabolize myricyl palmitate (beeswax), tubercle wax, and tributyrin. All cultures except one produced positive results varying only in rate and extent to which they attacked these substrates. A pronounced lag phase in utilization of substrate by 3 microorganisms tested was attributed to saturation of enzyme sites with endogenous substrates. 2) With the exception of *Candida albicans* intact cells did not show esterase activity. However, positive results were obtained with extracts which suggested that deesterification was an initial step in utilization of waxes as sources of energy for the various microorganisms tested.

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Action of N,N'-diphenyl-p-phenylenediamine in Tocopherol Deficiency Diseases.* (24485)

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The ability of certain synthetic antioxidants, notably N,N'-diphenyl-p-phenylenediamine (DPPD), to delay or prevent appearance of Vit. E deficiency symptoms, including muscular dystrophy and sterility, has been demonstrated. This activity generally has been attributed to conservation of traces of Vit. E present in the lipid portion of diet and in tissues of experimental animals. In conformity with this view, Shull and associates(1) found that DPPD and other synthetic antioxidants delayed but did not prevent eventual occurrence of muscular dystrophy in guinea pigs maintained on tocopherol-deficient diet. However, experiments with rats(2) showed that fertility could be partially restored in sterile females even when Vit. E was rigorously excluded from the regenerative diet. In the present study it has been found that DPPD has no conservational effect upon Vit.

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