contain both a cyclic AMP–dependent protein kinase and a hormone-sensitive adenyl cyclase. All the effects of cyclic AMP have been postulated to be mediated via stimulation of protein kinase (*9*). If this hypothesis is correct, then substances that increase the cyclic AMP levels should increase phosphorylation of endogenous proteins, provided that the protein kinase is not already maximally stimulated. Cyclic AMP–dependent phosphorylation of proteins has been shown to include not only histones but a number of other proteins, including phosphorylase kinase (*10*) and ribosomal protein (*11*). The presence of both adenyl cyclase and protein kinase activities in these established cultured cell lines may provide a useful system for studying the interactions of hormones, adenyl cyclase, and protein kinase as well as the regulatory role of cyclic AMP.

Note added in proof: After this manuscript was accepted, Perkins *et al.* (*12*) reported the presence of cyclic AMP–dependent protein kinase in cultured glial cells.

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Fatty Acid Ethyl Esters of Rhizopus arrhizus

Abstract. *Gas chromatographic and mass spectrometric analyses on selected lipid fractions revealed for the first time the presence of ethyl esters of long-chain fatty acids as biological products. Ethyl esters of oleic, palmitic, and stearic acids were detected in relative concentrations of 21.2, 2.4, and 1.5 percent, respectively, of the total methyl and ethyl ester fraction. Both saturated and unsaturated ethyl esters contain pronounced mass spectral fragments at a mass-to-charge ratio of 88.*

The presence of naturally occurring esters of short- and long-chain fatty acids has been documented for several plant and animal species. The complex epicuticular waxy coating of higher plant surfaces (*1*, *2*), insects (*3*, *4*), oils of certain aquatic animals (*5*), as well as the mandibular canal of the porpoise (*6*) are reported to contain waxy esters. Recently, methyl esters of long-chain fatty acids have been reported for corn pollen (*7*), chlamydospores of *Ustilago maydis* (*8*), and the sporangiospores and mycelia of *Rhizopus arrhizus* (*9*). The data in this report demonstrate for the first time the presence of novel esters of certain long-chain fatty acids containing ethyl groups.

Mycelial fragments (suspended in 0.5 ml of sterile distilled water) were inoculated onto Fothergill and Yeoman's solid medium (*10*) and grown in the dark at 25°C. After a 3-day incubation period the cultures were harvested. The mycelial mats were immediately frozen and lyophilized, and 0.434 g of the lyophilized material was extracted with a mixture of chloroform and methanol (3 : 1) followed by *n*-hexane as previously described (*9*). The freely extractable lipids were fractionated by silica gel column chromatography, and the fatty acid ester components were obtained in the neutral lipid fraction by elution with benzene. The individual methyl and ethyl esters were separated by gas-liquid chromatography, and structure confirmations were made by comparison with authentic standards by use of mass spectrometry and co-chromatographic techniques.

As previously reported (*9*), methyl esters ranging in carbon chain length from C_{14} to C_{24} were found in the neutral lipid fraction of total lipid extracts from *R. arrhizus*. The distribution of methyl esters detected under the conditions presented here is given in Fig. 1. No branched chain isomers were detected. After a 3-day growth period the significant methyl ester components were palmitate and oleate. Fluctuations in relative concentrations of these esters did occur with time but methyl palmitate and oleate remained the predominant saturated and unsaturated methyl esters. The esters of long-chain acids containing ethyl groups were also significant components (Fig. 1). The predominant ethyl esters correspond to the distribution of major free fatty acids typical for this organism (*9*). Ethyl oleate was the predominant ethyl component (21.2 percent) in the 3-day-old cultures, whereas ethyl palmitate (2.4 percent) and ethyl stearate (1.5 percent) were in lower concentrations. All values are expressed as the percentage of the total methyl and ethyl ester fraction. Relative concentration values changed significantly with age of the culture.

Standards for mass spectrometric comparison were prepared by treating the corresponding fatty acids with BF_3-ethanol or BF_3-methanol followed by extraction and gas chromatographic resolution as described previously for the preparation of esters (*9*). Approximately 85 percent of the gas chromatographic effluent was allowed to enter a Hitachi Perkin-Elmer RMU-6 by means of a glass separator operated at 250°C. The ion source was 270°C and scans were from 14.0 to 400 mass-to-charge ratio (m/e) in 10 seconds. Scans of the natural ethyl esters and the authentic standards produced equivalent spectra. For ethyl palmitate the rearrangement peak ($C_4H_8O_2^+$) at an m/e of 88 is the base peak, which is analogous to the pronounced m/e of 74 of straight-chain methyl esters. The fact that the base peak shifts from an m/e of 74 to an m/e of 88 in ethyl esters is due to the ethyl side chain being incorporated in the rearrangement ion formed by 2,3-cleavage. A methoxycarbonyl peak ($m/e = 101$) is also present. The molecular ion minus 45 ($M - 45$) in this spectrum and the $M - 43$ are both pre-

dominant in ethyl palmitate and have been reported as characteristic peaks for ethyl esters (*11*). Differences in relative intensities, particularly with respect to the molecular ion, were noted between our data and the values reported earlier by Ryhage and Stenhagen (*11*). However, the appearance of fragments such as *m/e* of 88, 157, 213, and at M − 45 and M − 43 are intense and apparent in both instances. Differences resulting in a lower contribution by the molecular ion may be attributed to different operational conditions and instrumental characteristics. The peak *m/e* of 88 and of 101 are predominant in the monounsaturated ethyl esters; however, as is the case for methyl oleate, an *m/e* of 55 is also the base peak for the ethyl ester. The M − 46 represents loss of ethanol from the monounsaturated ethyl ester and corresponds to the pronounced M − 32 observed for monounsaturated methyl esters (*12*). It seems possible that ethyl esters may be more common as natural products than one might expect because the peaks of *m/e* 88 and of 101 represent major fragments in not only α-methyl and β-methyl branched long-chain methyl esters (*13*) but also as shown here for long-chain ethyl esters.

The biological role (or roles) of either methyl or ethyl esters of long-chain acids is obscure at this time but their function may be more than as simple storage products. It has been reported that low concentrations of methyl esters, for example, will enhance auxin and gibberellin activity (*14*). Volatile constituents of oils containing both methyl and ethyl esters of only low molecular weight fatty acids (C_2 to C_8) have been reported with the use of gas chromatography retention data for various fruits, such as pineapple (*15*). The biosynthesis of methyl esters of long-chain acids has been demonstrated by a soluble enzyme from *Mycobacterium phlei* with the methylating agent being *S*-adenosylmethionine (*16*). Additional significance of this report is that there is no known mechanism to explain the transfer of ethyl groups required to synthesize the compounds we describe here. Methyl groups, for example, are commonly associated with "carrier" molecules such as tetrahydrofolic acid or *S*-adenosylmethionine. On the other hand, it has been reported that synthesis of high molecular weight waxy esters of cabbage involve the esterification of free, activated, or protein-bound fatty acids with fatty alcohols (*2*). Since there is an absence of free ethanol in plant systems or known ethanol-containing carrier molecules, an unknown esterification process may be in operation.

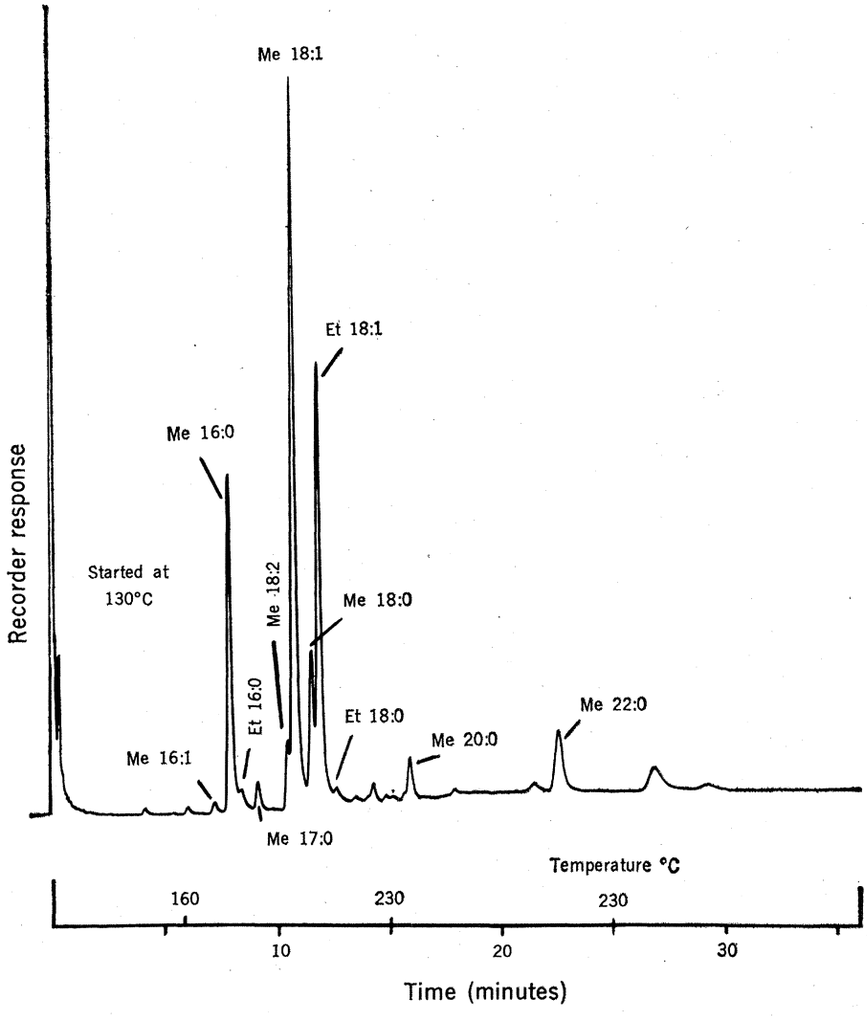

Fig. 1. Gas chromatographic separation of methyl and ethyl esters of long-chain fatty acids of *Rhizopus arrhizus* by use of a stainless steel capillary column (100 feet by 0.02 inch) coated with 3 percent Apiezon L. Injector temperature, 250°C; detector temperature, 325°C; temperature program, from 130° to 230°C at 6°C per minute with a 2-minute initial hold. Approximately 85 percent of gas chromatographic effluent was allowed to enter a Hitachi Perkin-Elmer RMU-6 mass spectrometer by means of a glass separator operated at 250°C. The ion source was at 270°C and scans were from 14.0 to 400 *m/e* in 10 seconds.

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