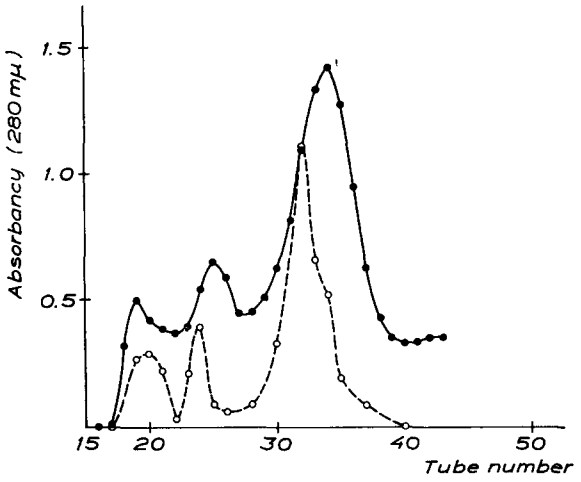

Fig. 2. Distribution of protein and of cellulase activity after gel filtration of an enzymic preparation from *Penicillium notatum* on Sephadex G–100. ●—●, absorbancy at 280 mμ; ○- - - -○, cellulase activity measured against CM-cellulose.

be found. Behind the last active peak a brown coloured material migrated, not shown in the diagram.

From the experiments made so far using gel filtration it appears that fungi produce multiple forms of cellulase and that the components may vary widely in molecular size. However, as will be published later, it seems that the multiple cellulase system of *P. notatum* consists of a common low molecular weight enzymic component associated with different inactive substances.

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Studies on lipolytic enzymes
II. The effects of *n*-butyl carbamic acid methyl ester on hydroxamic acid synthesis catalyzed by canine-liver lipase

It has been reported previously by us[1] that BCME is a remarkably potent species-specific inhibitor of canine liver and kidney lipase (EC 3.1.1.3) both *in vitro* and *in vivo*.

Abbreviation: BCME, *n*-butylcarbamic acid methyl ester.

Inhibition kinetics were described for both liver and kidney enzymes catalyzing the hydrolysis of tributyrin, an essentially insoluble substrate. LIPMANN AND TUTTLE[2] demonstrated that a characteristic of esterolytic enzymes is their ability to catalyze the synthesis of hydroxamic acids from hydroxylamine and a free fatty acid. As these substrates, under alkaline conditions, are both soluble it became of interest to determine the inhibition kinetics of BCME in the hydroxamic acid synthesis-reaction catalyzed by canine-liver lipase.

Aqueous extracts of dog-liver acetone powders were prepared by extracting 1 g of powder with 10 ml of cold water for 10 min in a polypropylene centrifuge tube and then centrifuging at 34 000 $\times$ g for 5 min. The clear supernatant fluid was used as such without further fractionation. The usual yield of supernatant fluid was 7.5–8 ml containing 300–350 g of soluble protein.

The kinetics of inhibition of the hydroxamate-synthesis reaction, induced by BCME, were studied using essentially the conditions and analytical methods described by LIPMANN AND TUTTLE[2]. In all cases, the enzyme was pre-incubated with a constant concentration of either hydroxylamine or sodium octanoate for 5 min before limiting amounts of the other substrate were added. Where BCME was a component of the reaction mixture, this was added before pre-incubation. This procedure was used to ensure that the enzyme complexes formed would be at steady states and would be preferentially utilized during the initial course of the reaction.

The data from kinetic studies were plotted using the methods of LINEWEAVER AND BURK[3] and curves were fitted by least-square calculations using an IBM 1620 computer programmed for these computations. The following notations were used:

E_o = moles of active center of the enzyme initially present
E = unassociated enzyme
I = moles of inhibitor added.

Initial rate studies utilizing various amounts of hydroxylamine (H), octanoate (T), and BCME (I) (Figs. 1 and 2) suggest the following mechanisms for the hydroxamic acid synthesis:

$$E + H \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EH$$

$$EH + T \xrightarrow{k_{+2}} E + \text{hydroxamic acid}$$

$$EH + I \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} EHI$$

with the conservation equation of $E_o = [E] + [EH] + [EHI]$,
where EH = hydroxyl amine–lipase complex and EHI = inhibited EH complex.

Using the KING-ALTMAN method[4] of solving the equations, the inhibited velocity, v_i, of hydroxamic acid formation is:

$$k_{+2}E_o/v_i = (K_1/[H] + 1 + [I]/K_3)/[T] + k_{+2}/(k_{+1}[H]) \quad (1)$$

where

$$K_1 = k_{-1}/k_{+1} \text{ and } K_3 = k_{-3}/k_{+3}$$

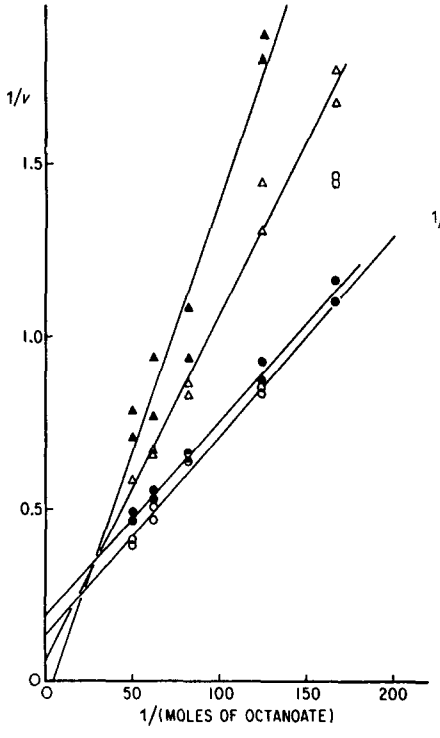

Fig. 1. BCME inhibition of hydroxamic acid synthesis with the liver lipase preincubated with hydroxylamine. ▲, $5.0 \cdot 10^{-4}$ M BCME (-0.044 ± 0.262); △, $2.0 \cdot 10^{-4}$M BCME (0.051 ± 0.106); ●, $1.0 \cdot 10^{-4}$ M BCME (0.192 ± 0.039); ○, no BCME (0.134 ± 0.055). ———, least-square line. Figures in parentheses are intercept ± 95% confidence limits.

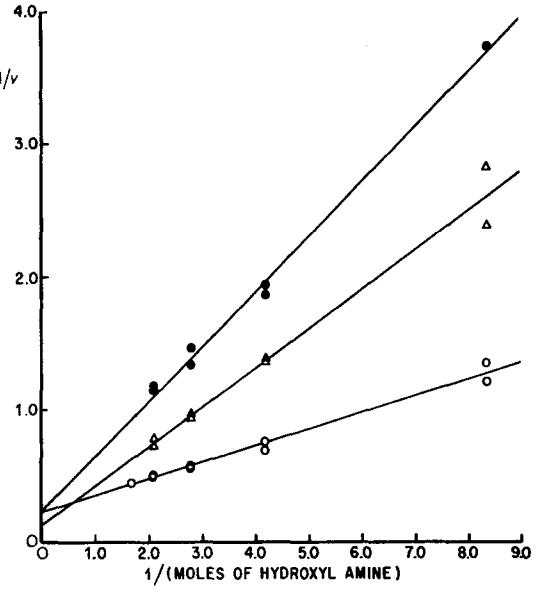

Fig. 2. BCME inhibition of hydroxamic acid formation from preincubated dog-liver lipase and octanoate. ●, $2.0 \cdot 10^{-4}$ M BCME (0.250 ± 0.120); △, $1.5 \cdot 10^{-4}$ M BCME (0.127 ± 0.223); ○, no BCME (0.229 ± 0.061). ———, least-square line. Figures in parentheses are intercept ± 95% confidence limits.

Using the least-square method developed by BAKER AND MAHLER[5] the constants calculated from experiments in which preincubation with various concentrations of hydroxylamine and BCME were performed (Fig. 1) were:

$$K_1 = 0.14\ \text{M};\ k_{+2}/k_{+1} = 100;\ \text{and}\ K_3 = 3.0 \cdot 10^{-4}\ \text{M}.$$

LINEWEAVER–BURK plots of the inhibited and uninhibited rates for fixed octanoate concentrations show common intercepts for large hydroxylamine concentrations (Fig. 2). The common intercept would result if preincubation of octanoate with lipase resulted in an octanoate–lipase complex and this complex was inhibited by BCME, according to the model:

$$E + T \underset{k_{-1'}}{\overset{k_{+1'}}{\rightleftharpoons}} ET$$

$$ET + H \xrightarrow{k_{+2'}} E + \text{hydroxamic acid}$$

$$ET + I \underset{k_{-3'}}{\overset{k_{+3'}}{\rightleftharpoons}} ETI$$

with the conservation $E_o = [E] + [ET] + [ETI]$.

Assuming steady state conditions, the inhibited velocity, v_i, can be shown (by the KING-ALTMAN method[4])to be:

$$1/v_i = \{(K_{1'}/[T] + 1 + [I]/K_{3'})/[H] + k_{+2'}/(k_{+1'}[T])\}/k_{+2'}E_o \equiv \Phi_o + \Phi_2/[H] + \Phi_{12}[I]/[H] \quad (2)$$

where

$$K_{1'} = k_{-1'}/k_{+1'},\ K_{3'} = k_{-3'}/k_{+3'},\ \Phi_o = k_{+2'}/(k_{+1'}[T]k_{+2'}E_o)$$
$$\Phi_2 = (K_{1'}/[T] + 1)/(k_{+2'}E_o),\ \text{and}\ \Phi_{12} = (1/K_{3'})/(k_{+2'}E_o)$$

Estimates of the octanoate binding constants, $K_{1'}$, and the inhibitor binding constant, $K_{3'}$, could be made if $k_{+1'}/k_{+2'}$ were known. Such data are not available, however, but some estimates about the upper limit for $k_{+1'}/k_{+2'}$ and $K_{3'}$, the inhibition binding constant, can be approximated.

Since $\Phi_2/\Phi_{12} = (K_{1'}/[T] + 1)/(1/K_{3'})$, an estimate of $K_{3'}$ can be made if $0.02 > K_{1'} > 0$, then $K_{3'} < (3 - 7) \cdot 10^{-5}$. Any value of $K_{1'} > 0$ would give a smaller value than $7 \cdot 10^{-5}$ for $K_{3'}$. Thus, the value of the constant for the binding of BCME ($K_{3'}$) by the octanoate–lipase complex is at least a factor of 4 smaller than that (K_3) for binding of BCME to the hydroxyl amine–lipase complex.

As $\Phi_2/\Phi_o = (K_{1'}/[T] + 1)/(k_{+2'}/k_{+1'}[T])$, the data indicate that the value for $k_{+1'}/k_{+2'} < 69$ for $K_{1'} > 0$ and thus the speed of attachment of hydroxyl amine and/or octanoate to the complex (formed with the complementary substrate) differs from their (or its) speed of attachment to the enzyme.

LIPMANN AND TUTTLE[2] proposed a mechanism for hydroxamic acid synthesis catalyzed by liver lipase which involved obligatory primary attachment of the fatty acid substrate to the catalytic site of the enzyme with the formation of an acid anhydride. This anhydride was then postulated to react spontaneously with an excess of hydroxylamine present, to form the hydroxamic acid.

The studies presented in this report, contrary to the mechanism proposed by LIPMANN AND TUTTLE[2], are consistent with the fatty acid and the hydroxylamine both being substrates for the enzyme, either being able to attach to the catalytic site first. This mechanism is consistent with the LINEWEAVER–BURK plots of inhibited and uninhibited velocities having a common intercept for very large substrate (hydroxylamine or fatty acid) concentrations.

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