in both thymuses (13 of 69). In earlier studies bilateral thymic tumor evolution was rare following *intraperitoneal* virus inoculation(6). If these cases of bilateral thymic tumor in this study represent bilateral *evolution,* rather than unilateral evolution and subsequent direct spread, then a possible explanation may be leakage of virus during the intrathymic inoculation.

While this experiment was not designed to determine the latent period between inoculation and tumor development, it was noted that generalized lymphomas were already present in many cases by 55 days of age. This finding suggests that the latency of lymphoma following intrathymic inoculation is significantly shorter than that observed following intraperitoneal inoculation.

The consistent occurrence of tumors in that thymus which had been inoculated with the virus supports the view that the mechanism of viral leukemogenesis is a direct one.

If the mechanism is direct, it remains to explain the consistent *unilateral* evolution of thymic lymphoma following intraperitoneal inoculation where presumably both thymuses are exposed to virus. Two possibilities may be considered: the virus was able to enter only one thymus, or virus could enter both thymuses but one thymus was physiologically more responsive than the other. To evaluate the first possibility we are at present determining the time sequence for appearance of virus in each of the 2 thymuses. Preliminary assay results have indicated that following intraperitoneal inoculation, the amount of infectious virus in each of the 2 thymuses differs considerably, and preliminary electron microscopic studies support this observation (9).

Summary. Murine Leukemia virus (Rich) was inoculated into only one of the 2 thymuses of infant mice which were sacrificed 2 months later. The animals were analyzed to compare the incidence of lymphoma evolving in the inoculated thymus with the incidence of lymphoma evolving in the uninoculated thymus. It was found that of 30 animals suitable for analysis, in 90% of cases the tumor arose in the inoculated thymus, supporting the view that the mechanism of viral leukemogenesis is *direct.*

We thank R. Clymer, W. Harrell, and S. Siegel for skilled technical assistance.

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Received February 16, 1966. P.S.E.B.M., 1966, v122.

Pancreatic Lipase Activity in Deuterium Oxide.* (31173)

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In most of the studies on enzymatic activity in D_2O, soluble substrates have been employed. However, there are a large number of enzymes that act on insoluble substrates, for which the conventional Michaelis-Menten kinetic analysis is inappropriate(1). One of these is pancreatic lipase (glycerol ester hydrolase, EC 3.1.1.3), shown by Sarda and Desnuelle(2) to act only on emulsified esters; although crude preparations could hydrolyze

* This work was supported by U. S. Atomic Energy Commission.

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water-soluble compounds such as triacetin, the highly purified enzyme had almost no effect on this substrate unless it was present in excess of its solubility.

Thus pancreatic lipase seemed to be an interesting enzyme on which to study solvent effects of deuterium oxide. Apparently the only previous work with this system was carried out in 1934 with a crude preparation (3); hydrolysis of triolein was not markedly affected by 30% D_2O.

Methods. Crude porcine pancreatic lipase (Sigma Chemical Co.) was purified by the method of Baskys *et al*(4). Paper electrophoresis of the final product revealed one major and one rather diffuse minor component. Although the dried enzyme appeared to be stable when stored in the cold, aqueous solutions deteriorated rather rapidly; addition of glycerol, cysteine, or deoxycholate did not prevent the loss of activity. Thus each day a sample of enzyme was weighed and dissolved in water. The solution was divided into a number of 0.5 or 1.0 ml samples which were frozen until immediately before use.

The enzyme was active against emulsified glycerol triesters of acetic, propionic, butyric, and oleic acid. Hydrolysis of triolein was very slow unless sodium deoxycholate was added to the reaction mixture(2,4). The activity with triacetin in solution was virtually negligible, an indication that non-specific "esterases"(2) were not present in the purified enzyme.

Tributyrin was used as the substrate in most assays. Emulsions were prepared in 10% gum arabic according to Sarda and Desnuelle(2); generally, 3 ml of tributyrin were blended with 20 ml gum arabic solution. Other emulsifying agents or detergents (Triton X-100, BRIJ-98, WR-1339, Duponal C, sodium taurocholate) proved to be unsatisfactory; at the concentrations necessary to stabilize the emulsions, they inhibited the enzyme.

The number of particles per unit volume was counted in a Coulter counter; the average number was 1.43×10^9 particles/ml, and the total lipoid surface area was calculated to be about 140 cm^2/ml, although the distribution of particle sizes was so broad that this figure is only an approximation. The total number of particles and their distribution appeared to be identical in both H_2O and D_2O.

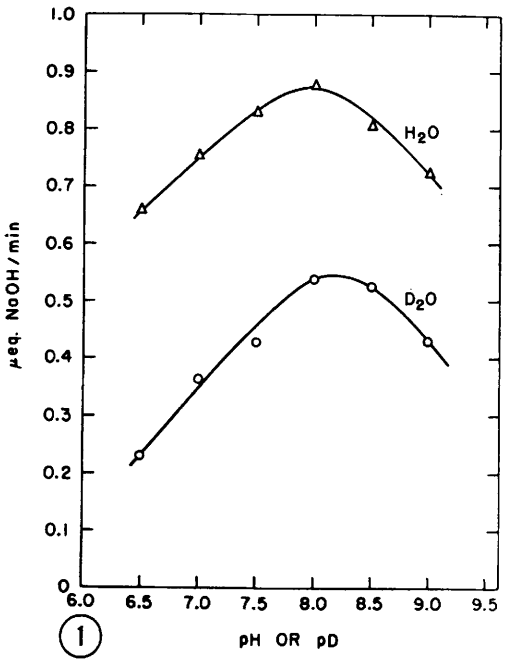
Lipase activity was measured titrimetrically. An automatic titration apparatus(5) was converted to a pH-stat so that the acid formed by hydrolysis of the tributyrin was continuously neutralized to a predetermined pH. With 0.005 N NaOH as the titrant, the fluctuation in pH was about 0.1 unit in the course of an assay. In the experiments with D_2O as solvent, 0.005 N sodium hydroxide in D_2O of the same concentration as in the reaction mixture was used for titration to avoid dilution of the solvent with H_2O. The rate of hydrolysis was linear for at least 15 minutes under the conditions employed. All experiments were carried out at 25°C. Non-enzymatic hydrolysis of tributyrin was negligible.

In most assays, a quantity of the tributyrin emulsion in gum arabic (0.2 to 5.0 ml) was introduced into the reaction vessel of the pH-stat and diluted with water (or D_2O) to 10 ml. The pH was adjusted to the predetermined value, then the reaction was started by addition of 0.1 to 0.5 ml of the enzyme solution. The amount of NaOH added was noted at one-minute intervals for 15 minutes. The slope of the plot of volume of NaOH against time was calculated by the method of least squares. The results are expressed as microequivalents of NaOH added per minute.

Results. Fig. 1 shows that in H_2O the pH for optimum lipase activity is about 7.9; in D_2O, the optimum pD is about 8.2. The pD values were obtained by adding 0.4 to the readings on the Beckman Model G pH meter (6).

The influence of 99.6% D_2O as solvent is shown in Fig. 2. At pH or pD 8.0, near the optima for both solvents, lipase activity was reduced by D_2O to about 46% of control activity. Data at pH 7.5 and pD 8.5 are shown on the same Figure.

The effect of varying concentrations of D_2O was studied at 4 different substrate concentrations (Fig. 3). At the 2 lower concentrations (0.09 and 0.22 millimoles tributyrin)



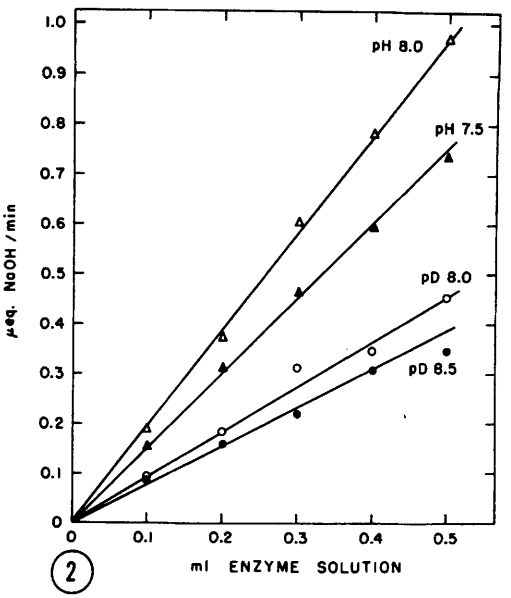


FIG. 1. Optimum pH and pD for purified pancreatic lipase. Reaction mixtures contained 0.9 millimole of emulsified tributin 10 ml.

FIG. 2. Hydrolysis of tributyrin in H_2O (triangles) and D_2O (circles). Reaction mixtures contained 0.9 millimole of substrate in 10 ml.

there was little inhibition even at high concentrations of D_2O. The inhibitory effect of D_2O was much more striking at the higher concentrations (0.90 and 2.24 millimoles). Estimates of the maximum velocities (at infinite substrate concentration) in H_2O-D_2O mixtures suggested that there was probably a linear relationship between D_2O concentration and inhibition of lipase amounting to about 0.7%/% D_2O (Table I). These estimates are not very precise because only 4 points were used in calculating each value.

Discussion. It would appear from the fact that the rate of hydrolysis increases linearly

TABLE I. Effect of D_2O Concentration on Maximum Velocity of Pancreatic Lipase.

Conc D_2O	V_{max}	% Control activity
0	33.1 ± 3.3*	100
25	28.3 ± 6.2	85
50	23.0 ± 4.7	69
75	16.3 ± 1.6	49
100	10.8 ± 1.0	33

* Mean ± S.E., calculated from plots of velocity^{-1} *vs* substrate concentration$^{-1/3}$.

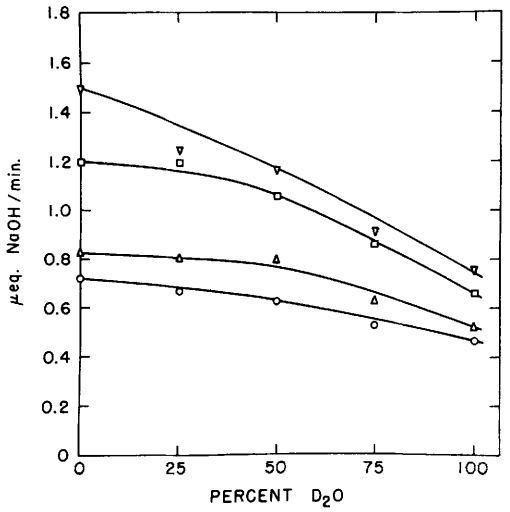


FIG. 3. Effect of varying concentrations of D_2O on hydrolysis of tributyrin at 4 levels of substrate: 0.09 (○), 0.22 (△), 0.90 (□), and 2.24 (△) millmoles in 10 ml, corresponding to surface areas of 28, 70, 280, and 700 cm^2. Experiments were carried out at pH 8 or pD 8. Points at 25, 50, and 75% D_2O are averages of duplicates; those at 0 and 100% are averages of triplicates.

with increasing enzyme concentration (Fig. 2), rather than as a function of the ⅔ power of the concentration, that the kinetics of pancreatic lipase probably do not satisfy the Gyani-Freundlich isotherm as applied by McLaren(1).

Benzonana and Desnuelle(7) have recently studied lipase activity as a function of particle size of the substrate, in this case long-chain triglycerides emulsified with bile salts. Their data are consistent with a mechanism described by the rate equation:

$$\frac{1}{v} = \frac{1}{V_m}(1 + KA_o/I)$$

where v is the reaction velocity observed with substrate interfacial area I, V_m is the maximum velocity, A_o is the volume of the emulsion, and K is a combination of constants, analogous to the Michaelis constant.

Our data do not fit the above equation, a plot of 1/v against 1/I yielding a curve that is concave to the abscissa.‡ Benzonana and Desnuelle(7) also failed to obtain linear plots of data obtained with emulsions prepared with gum arabic. However, as indicated above, estimates can be made of the maximum velocities at different concentrations of D_2O, even though the constant in the preceding equation cannot be evaluated.

It is clear that D_2O has an appreciable inhibitory action; however, at lower concentrations of substrate the inhibition is nearly negligible. This observation suggests that there is a greater effect of D_2O on the rate constants for enzyme-substrate binding than on those for hydrolysis. In the case of homogeneous enzymatic reactions, a larger effect of deuterium on the Michaelis constants than on the maximum velocities means that a normal isotope effect will decrease as the concentration of substrate decreases, and will become inverse at low substrate concentrations(8). It is reasonable to believe that a similar explanation is tenable in the case of a soluble enzyme acting upon an insoluble substrate.

‡ As indicated in Table I, linear plots are obtained when 1/v is plotted against $1/I^{-⅛}$. However, this approach seems mechanistically unreasonable.

Summary. The activity of purified pancreatic lipase, with tributyrin emulsified in gum arabic as the substrate, has been studied in ordinary water and in varying concentrations of D_2O up to 100%. Estimates of maximum reaction velocities indicate that the degree of inhibition increases linearly with increasing D_2O. At low substrate concentrations, however, the inhibition produced by D_2O was much less, an indication that there is a stronger solvent effect on enzyme-substrate binding than on the rate of hydrolysis itself.

We are indebted to William J. Eisler for modification of the pH-stat, to Herbert E. Kubitschek for counting the particles in the emulsion, and to Joseph J. Katz and Henry L. Crespi (Chemistry Division) for supplies of 99.8% D_2O.

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Received February 17, 1966. P.S.E.B.M., 1966, v122.