absence of –OH groups in the polymer suggest that it may involve hydrogen transfer, *e.g.*,

$$R—O—O—H + CH_2{=}CHC_6H_5 \longrightarrow R—O—O\cdot + CH_3—\dot{C}H(C_6H_5) \quad (9)$$

such a scheme yields, as an over-all rate expression

$$\frac{-d[M]}{dt} = k_p[M]^{3/2}\left(\frac{k_i[P]}{k_t}\right)^{1/2} \quad (10)$$

The 3/2-order reaction in respect to monomer agrees with the data on individual runs (Figs. 2 and 3) but not with the plots of Fig. 3. Contributions from an initiation process of higher order in monomer would improve the agreement, *i.e.*, from a termolecular initiation reaction.[31]

Another alternative would be through the introduction of a second termination process

$$P\cdot + M\cdot \xrightarrow{k_t'} \text{polymer} \quad (8f)$$

This leads as a limiting value, to a rate expression

$$\frac{-d[M]}{dt} = k_p[M]^2\left(\frac{k_i k_r}{k_t' k_{tr}}\right)^{1/2} \quad (11)$$

although the calculations in our discussion of the transfer reaction based upon $\bar{P}$ and the known relation between polymerization rate and the rate of reaction 8e show that normal termination by polymer chain coupling must necessarily account for at least 25–50% of all the termination reactions in our kinetic runs. Reaction 8f merits further discussion. At first it may appear unlikely that, in a system where chain transfer occurs only once in every several hundred chain propagation steps, the concentration of P· radicals will be high enough to figure in the termination process. However, studies of the polymerization of styrene in the presence of oxygen, which leads to the formation of a linear polyperoxide by the sequence

$$\sim M\cdot + O_2 \longrightarrow \sim M—O—O\cdot \quad (12a)$$

$$\sim M—O—O\cdot + M \longrightarrow \sim M—O—O—M\cdot \quad (12b)$$

shows a pronounced drop in over-all rate of styrene disappearance as the amount of oxygen in the polymer increases.[32] This, in turn, suggests that reactions of the type of 8d above are slow compared with ordinary chain propagation, *i.e.*,

$$k_p > k_r$$

Further, termination reactions between unlike radicals often show enhanced rates; but, even taking both of these factors into consideration, it seems unlikely that reaction 8f can account for more than a portion of the chain terminations.

Intrusion of 8f into the kinetic scheme would also alter the picture of the peroxide transfer reaction, since now every transfer results in the termination of *two* chains. Accordingly transfer constants measured by either of the methods discussed would be too large by a factor of two if every transfer were to end a chain and, in practice, may be somewhat high.

Since peroxide decomposition occurs by two different processes related to monomer consumption, which is already complex, it is probably best treated as in the preceding section without attempting to derive time-dependent kinetic expressions, particularly since significant quantities apparently disappear by other processes—either polar or radical—which are not taken into account in our reaction scheme. In fact, it is doubtful if any of the empirical expressions for hydroperoxide disappearance in the presence of olefins are of much theoretical significance at this time.

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(31) Recent evidence indicates that the thermal initiation of styrene polymerization is itself a termolecular process, *cf.* F. R. Mayo, THIS JOURNAL, **75**, 6133 (1953).

(32) S. Medvedev and P. Zeitlin, *Acta Physicochim. U.R.S.S.*, **20**, 3 (1945).

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Enzyme-catalyzed Exchange of Oxygen Atoms between Water and Carboxylate Ion[1]

BY RONALD BENTLEY[2] AND D. RITTENBERG[3]

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The hydrolysis of acetylcholine and ethyl acetate catalyzed by acetylcholinesterase, and the alkaline hydrolysis of acetylcholine have been shown to involve splitting of the –CO–O– bond. Acetylcholinesterase has been found to catalyze the exchange reaction between the oxygen atoms of fatty acids and water: $RCO_2{}^{16}H + 2H_2O^{18} = RCO^{18}{}_2H + 2H_2O^{16}$. The extent of the exchange is greatest with acetic acid, diminishes with propionic and formic acid, and is least pronounced with butyric acid. Similarly, two lipase preparations have been shown to catalyze the same general reaction, but in this case the extent of exchange was greatest with butyric acid. The nature of the activation process involved in esterase action is discussed in relation to these results.

Although enzyme action is generally interpreted in terms of a reactive enzyme–substrate complex, little is known of the process leading to formation of the initial complex. In an investigation of the nature of this activation process, Sprinson and Rittenberg[4] observed that chymotrypsin brought about the exchange of the carboxyl oxygen atoms of carbobenzyloxy-L-phenylalanine with the oxygen atoms of the solvent, water. The exchange did not take place in the absence of the enzyme. Similarly,

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(4) D. B. Sprinson and D. Rittenberg, *Nature*, **167**, 484 (1951).

when L-leucine containing N^{15} and D in the α-position was incubated with a pig heart preparation (containing transaminase) the recovered leucine was found to have lost 98% of the deuterium but no N^{15}. It was suggested that such reactions, occurring under conditions where the usual reaction associated with the enzyme did not take place, should be called *virtual reactions*. A similar chymotrypsin-catalyzed reaction, between water and acetyl-3,5-dibromo-L-tyrosine was reported by Doherty and Vaslow.[5] Stein and Koshland[6] have described an exchange of oxygen atoms between KH_2PO_4 and H_2O^{18} catalyzed by alkaline phosphatase.

The reactions brought about by acetylcholinesterase have been extensively studied[7] and Wilson[8] has shown that acetylcholinesterase catalyzed the hydrolysis of thiolacetic acid; he suggested that in a medium of H_2O^{18} the enzyme would bring O^{18} exchange in acetic acid.

We here report on the ability of acetylcholinesterase and of two lipases to catalyze exchange reactions between some carboxylic acids and H_2O^{18}.

Experimental

Acetylcholinesterase.—The following enzyme preparations were used: A highly active preparation of acetylcholinesterase (4 g. of acetylcholine hydrolyzed per hr. per ml.) was supplied to us by Dr. Nachmansohr. We are indebted to him for this kindness. Thirty ml. of the enzyme solution was treated with 3 ml. of 0.05 *M* phosphate buffer, *p*H 8, and then dialyzed overnight against 0.05 *M* phosphate buffer, *p*H 8, at 4°. After further dialysis against distilled water at 4° the solution was lyophilized to yield 134 mg. of a white, flaky material.

Lipases.—Two preparations were obtained through the kindness of Dr. Kaunitz, designated as lipase A and B. Lipase A, originally obtained from Wilson and Co., was quoted as having a *p*H optimum of 8 and a temperature optimum of 40°. Lipase B, a Rohm and Haas product (lipase B, No. 2) had *p*H optimum of 6 and temperature optimum of 37°.

Reaction mixtures were prepared by evaporating an appropriate buffer solution (usually 5–10 ml.) to dryness. Final traces of water were removed in high vacuum and the residue redissolved in H_2O^{18} (unless otherwise stated 1.27 atom per cent. excess O^{18}, usually 2–3 ml.).[9] The sodium salt of the fatty acid and the enzyme preparation were added and the mixture was incubated under the desired conditions. The reaction was stopped by rapid chilling and addition of 0.5 ml. of ice-cold 5 *N* sulfuric acid. Water and free fatty acid were separated from inorganic salts by distillation in vacuum in a closed system, using a receiver cooled in ethanol-CO_2. The silver salt of the fatty acid was prepared and decarboxylated for mass-spectrometric analysis as previously described.[10] Pilot experiments demonstrated that silver propionate and silver *n*-butyrate were decarboxylated smoothly and at a lower temperature than was needed for silver acetate. The decarboxylation was carried out in one leg of a vacuum tube of the type described by Sprinson and Rittenberg.[11]

(5) D. G. Doherty and F. Vaslow, THIS JOURNAL, **74**, 931 (1952).

(6) S. S. Stein and D. E. Koshland, *Arch. Biochem. Biophys.*, **39**, 229 (1952).

(7) I. B. Wilson and F. Bergmann, *J. Biol. Chem.*, **185**, 479 (1950); I. B. Wilson and F. Bergmann, *ibid.*, **186**, 683 (1950); F. Bergmann, I. B. Wilson and D. Nachmansohn, *ibid.*, **186**, 693 (1950); I. B. Wilson, F. Bergmann and D. Nachmansohn, *ibid.*, **186**, 781 (1950).

(8) I. B. Wilson, *Biochim. Biophys. Acta*, **7**, 520 (1951).

(9) The H_2O^{18} was obtained from the Stuart Oxygen Co., San Francisco, on allocation of the Atomic Energy Commission.

(10) R. Bentley, THIS JOURNAL, **71**, 2765 (1949).

(11) D. B. Sprinson and D. Rittenberg, *J. Biol. Chem.*, **180**, 707 (1949).

Results[12]

Mechanism of Hydrolysis of Acetylcholine.—In preliminary experiments, the hydrolysis of acetylcholine in H_2O^{18} was carried out with alkali and acetylcholinesterase, to establish the validity of the technique. Since acid hydrolysis proceeds only slowly (at *p*H 3.6 and 100° the extent of hydrolysis of acetylcholine is only 8.9 per cent. in 9 hours[13]), this was not attempted in H_2O^{18} because of the rapid, acid-catalyzed exchange reaction of acetic acid.[8]

(a) **Alkaline Hydrolysis.**—Acetylcholine chloride (100 mg.) and sodium hydroxide (59.9 mg.) in 2 ml. of H_2O^{18} were kept for ten minutes at room temperature; atom per cent. excess O^{18} in isolated silver acetate, 0.62; per cent. of oxygen atoms derived from water = 49.

(b) **Enzymatic Hydrolysis.**—Acetylcholine chloride (200 mg.) and a dried buffer mixture, *p*H 7, (prepared by evaporation of 10 ml. of a 0.13 *M* phosphate buffer) were dissolved in 2 ml. of H_2O^{18}, acetylcholinesterase (0.5 mg.) (the dried residue obtained by lyophilization of 0.5 ml. of a dilute preparation) was added and the mixture incubated for 90 minutes at 35°; atom per cent. excess O^{18} of recovered silver acetate, 0.62; per cent. of oxygen atoms derived from water = 51. In another experiment, acetylcholine chloride (200 mg.) and dried buffer mixture, *p*H 9.5 (prepared by evaporation of 10 ml. of a 0.2 *M* borate buffer) were dissolved in 2 ml. of H_2O^{18}. Acetylcholinesterase (0.2 ml. of the original preparation) was added and the mixture incubated for one hour at 35°; atom per cent. excess O^{18}, 0.53; per cent. of oxygen atoms derived from water = 46.

Mechanism of Enzymatic Hydrolysis of Ethyl Acetate.—Ten ml. of a 0.05 *M* phosphate buffer, *p*H 7, was evaporated to dryness, redissolved in 2 ml. of H_2O^{18} and 0.17 ml. of ethyl acetate and acetylcholinesterase (0.3 ml. of the original preparation) added. After incubation for 1 hour at 37°, volatile materials were distilled off *in vacuo*, the residue was acidified with 0.5 ml. of 0.5 *N* sulfuric acid and silver acetate prepared in the usual way (16.1 mg.); atom per cent. excess O^{18}, 0.60; per cent. of oxygen atoms derived from water = 52.

Exchange with Sodium Acetate.—One experiment will be described in detail, in particular to emphasize that the enzyme (acetylcholinesterase) remained active throughout such experiments. Two portions (5 ml.) of 0.05 *M* phosphate buffer, *p*H 5.5, were dried in the usual way. One portion was dissolved in H_2O^{18} (3 ml.); anhydrous sodium acetate (75 mg.) and lyophilized acetylcholinesterase (27.4 mg.) were then added. The solution now had *p*H 6.0. The second portion, to serve as a control, was made up similarly but without the addition of enzyme. After 18 hours at room temperature (20°), 0.2 ml. of the mixture to which acetylcholinesterase had been added was removed. This was diluted to 1.0 ml. with water, and added to a solution of acetylcholine chloride (100 mg.) in 9 ml. of 0.05 *M* phosphate buffer, *p*H 6.5; 0.5-ml.

(12) Where the percentage of oxygen atoms derived from the solvent has been calculated, suitable corrections have been made for any dilution with exchangeable oxygen atoms, or by addition of normal water.

(13) E. Hofmann, *Helv. Chim. Acta*, **13**, 138 (1930).

portions were withdrawn immediately and at ten-minute intervals, added to 20% trichloroacetic acid (1 ml.) and made up to a volume of 10 ml. One-ml. portions were used for acetylcholine analysis by the method of Hestrin.[14] The activity of the remaining enzyme was such that after 30 minutes, no acetylcholine remained in the mixture as shown by the following figures: Calculated for the original volume of test solution the observed values expressed as mg. of acetylcholine were: t_0, 81; t_{10}, 34; t_{20}, 12; t_{30}, 0. Isotope analysis of silver acetate isolated from the bulk of the solution and from the control experiment gave the following results

Acetylcholinesterase expt., atom per cent. excess O^{18}	1.19
Control experiment, atom per cent. excess O^{18}	0.02

i.e., under these conditions the reaction was 94% complete for the exchange of both oxygen atoms.

Effect of Time on the Reaction.—A second experiment was carried out under the same experimental conditions as in the previous case, except that the time of incubation was reduced to 4 hours; atom per cent. excess O^{18}, 0.45; per cent. exchange, 35.

Effect of *p*H on the Reaction.—The experimental conditions and the results obtained in studying the influence of *p*H on the enzyme-catalyzed exchange reaction are given in Table I.

TABLE I

EFFECT OF *p*H ON THE ENZYME CATALYZED EXCHANGE OF OXYGEN ATOMS BETWEEN ACETATE AND WATER

Experiment	*p*H[a]	Enzyme soln., ml.[b]	Sodium acetate, mg.	Atom % excess O^{18} in silver acetate isolated	Exchange, %
1	6.0	2	50	0.18	14
2	7.1	2	50	.10	7.9
3	8.0	2	50	.08	6.3

[a] 5 ml. of the buffer was evaporated to dryness and the residue dissolved in the enzyme solution taken for each experiment. The following buffer mixtures were used: water, 20 ml.; 0.2 *M* potassium acid phosphate, 5 ml.; 0.2 *N* NaOH—Expt. no. 1, 0.2 ml.; no. 2, 3.0 ml.; no. 3, 4.1 ml.; the *p*H given is that of the solution after addition of the sodium acetate. [b] The solution was prepared by dissolving acetylcholinesterase (22.5 mg.) in H_2O^{18} (6.2 ml.). Incubation of the reaction mixtures was carried out at 21° for 2 hours.

Effect of Varying Amount of Enzyme.—The effect of varying enzyme concentrations is shown in Table II.

TABLE II

EFFECT OF CONCENTRATION OF ENZYME ON REACTION BETWEEN ACETIC ACID AND H_2O^{18}

The reaction was carried out for 3 hours at 20° at *p*H 6.0.

Experiment[a]	Enzyme powder, mg.	Atom % excess O^{18} in isolated silver acetate	Cor. for control, atom % excess
A_1	0	0.059	...
A_2	1	.071	0.012
A_3	2	.083	.024
A_4	4	.135	.076
A_5	6.5	.181	.122
B_1	0	.051	...
B_2	4.0	.115	.064
B_3	24.5	.446	.395

[a] Different enzyme preparations were used in experiments A and B.

(14) S. Hestrin, *J. Biol. Chem.*, **180**, 249 (1949).

Effect on Other Fatty Acids.—In these experiments in which formate, propionate and butyrate were investigated the reaction mixtures were prepared from dried phosphate buffer, *p*H 5.5 (0.05 *M*, 5 ml.), 0.6 *mM* of the sodium salt of the fatty acid, and 2 ml. of a solution of acetylcholinesterase (29.8 mg.) in H_2O^{18} (6.2 ml.). The mixtures were incubated at 21°.

In the case in which sodium formate was employed as the substrate, 20 ml. of 0.2 *M* phosphate buffer, *p*H 5.4, was taken to dryness and the residue dissolved in 8.2 ml. of H_2O^{18}. The solution was divided into four equal portions and to the first were added 50 mg. of anhydrous sodium acetate and 9.1 mg. of acetylcholinesterase; to the second 50 mg. of anhydrous sodium acetate; to the third 50 mg. of sodium formate and 9.1 mg. of acetylcholinesterase; to the fourth 50 mg. of sodium formate. The four samples were kept at 20°. From the first two, silver acetate was isolated as described above. The third and fourth samples were neutralized to phenolphthalein with 1 *N* NaOH, evaporated to dryness and the residue extracted with hot absolute ethanol. The alcohol extract was evaporated to dryness and kept in a vacuum desiccator over $CaCl_2$ for 24 hours.

The silver acetate was decarboxylated thermally as described above. The sodium formate was mixed with 200 mg. of dry AgCl in a platinum crucible and rapidly pyrolyzed by the use of an induction furnace.[15] The results are given in Table III.

TABLE III

RATE OF ACTIVATION OF RELATED FATTY ACIDS BY ACETYLCHOLINESTERASE

Expt.	Substrate	Incubation time, hr.	Atom % excess O^{18} in acid	Atom % excess O^{18} cor. for control	Rate relative to acetate
1	Acetate	3	0.26	0.26	100
	Propionate	3	.12	.12	46
	Butyrate	3	.02	.02	8
2	Acetate	20	.89	.78	100
	Control without enzyme	20	.11		
	Formate	20	.45	.11	14
	Control without enzyme	20	.34		

Experiments with Lipase Preparations.—The results of experiments carried out at *p*H 8 are shown in Table IV and at *p*H 6.5 in Table V.

TABLE IV

EXCHANGE REACTIONS CATALYZED BY LIPASE PREPARATIONS AT *p*H 8

For each experiment 5 ml. portions of the following buffer mixture were evaporated to dryness: 0.2 *M* potassium hydrogen phosphate, 10 ml.; 0.2 *N* sodium hydroxide, 8.2 ml.; water, 40 ml. Tubes incubated at 37° for 15 hours.

Expt. no.	Substrate (mg.)	Enzyme (mg.)	H_2O^{18}, ml.	Atom % excess O^{18} in recovered fatty acid
1	Na acetate (60)	Lipase "A" (50)	2	0.03
2	Na acetate (60)	Lipase "B" (50)	2	.09
3	Na acetate (50)	.....	2	.04
4	Na butyrate (75)	Lipase "A" (50)	2	.73

(15) This method for the decarboxylation of some classes of compounds for O^{18} analysis will be described in another paper.

TABLE V

EXCHANGE REACTIONS CATALYZED BY LIPASE PREPARATIONS AT *p*H 6.5

For each experiment 5-ml. portions of the following buffer mixture were evaporated to dryness: 0.2 *M* potassium dihydrogen phosphate, 10 ml.; 0.2 *N* sodium hydroxide, 1.2 ml.; water, 40 ml. Each tube contained sodium *n*-butyrate (75 mg.), designated quantities of enzyme, and 2 ml. of H_2O^{18} (1.4 atom % excess). Incubation at 37° for 16 hours.

Expt. no.	Enzyme (mg.)	Atom % excess O^{18} in recovered silver *n*-butyrate
1	Lipase "A" (47.5)	0.93
2	Lipase "B" (53.4)	.02
3	Control; no enzyme	.05

Discussion

The alkali-catalyzed hydrolysis of acetylcholine proceeds by acyl–oxygen fission as is the case for the similar hydrolysis of other esters,[16] and β- and γ-butyrolactones.[17,18] Acetyl phosphate is also split at the C–O linkage[10] in alkaline solution. The reaction involves nucleophilic attack on the carbonyl carbon atom and probably proceeds through the intermediate (I).

$$(CH_3)_3{}^+NCH_2CH_2O\ldots\underset{\vdots\,OH^-}{\overset{\overset{O}{\|}}{C}}—CH_3 \qquad (I)$$

The acetylcholinesterase-catalyzed hydrolysis of both acetylcholine and ethyl acetate was also found to proceed with acyl–oxygen fission.[19] These observations are therefore in agreement with the hydrolytic mechanism first postulated by Wilson.[20] Subsequent attack of the intermediate by nucleophilic reagents (*e.g.*, H_2O) completes the hydrolysis. Substantially similar suggestions concerning the mechanism of esterase action have been postulated by other workers.[21–23]

Using amounts of enzyme which readily bring about the hydrolysis of acetylcholine, only one oxygen atom from the solvent water was introduced into acetic acid. However, with much larger quantities of enzyme it was possible to bring about a complete exchange of the oxygen atoms of acetic acid. Here we are not concerned with the hydrolysis which must introduce an atom of oxygen, but with the virtual reaction between acetic acid and the solvent. This latter reaction will lead to exchange of both oxygen atoms in acetate ion, and its occurrence confirms Wilson's prediction.[8]

Wilson concluded from his experiments with thiolacetic acid that the substrate was the undissociated acid.[8] The increase of rate of the virtual reaction as the *p*H is lowered (see Table I) similarly suggests that it is undissociated acetic acid which reacts with the enzyme. It is clear, however, that the rate is determined by additional factors since in passing from *p*H 8 to *p*H 6 the concentration of free acid increased by a factor of 100 whereas the reaction rate increased by little more than twofold.

The virtual reaction has all the characteristics of an authentic enzyme reaction, since in addition to the *p*H effect, the extent of exchange was proportional to the concentration of enzyme and to time. In one experiment, the action of acetylcholinesterase on acetic acid was investigated in D_2O. No D was introduced into the methyl group; apparently no activation of this part of the molecule takes place.

The same general activation of the carboxyl groups of fatty acids is caused by the two lipases we have studied. Lipase A is without action on acetate though it rapidly activates butyrate; lipase B activates acetate slightly but not butyrate (see Tables IV and V). It is striking that the mere presence of a $-CH_2-CH_2-$ group in passing from acetate to butyrate confers on lipase A the ability to activate the carboxyl oxygen atoms. It is difficult to believe that the usual van der Waals forces between the $-CH_2-CH_2-$ group and the enzyme can account for this great difference in the interaction of the enzyme with acetate and butyrate, respectively.

As in the case of acetylcholinesterase, it appears that we are dealing with a primary activation process and that the nature of the enzyme–substrate complex is very closely related not only for the hydrolytic and virtual reactions but also for the transesterification reaction catalyzed by esterases[24] and for the enzymatic hydrolysis of acid anhydrides observed by Wilson[25] and Bergmann, *et al.*[26]

In the case of lipases, little information is available about the nature of possible active sites, on which the substrates are bound. In this connection it may be significant that hog pancreatic lipase preparations have been reported[27] which lose activity on dialysis; the activity could be restored by the addition of L-histidine, maximum reactivation being obtained at a concentration of 0.1 *M*.

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