LXXXVII. ACTION OF ESTERASE IN THE PRESENCE OF ORGANIC SOLVENTS.

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Methods.

In the method of studying esterases elaborated by the author, the active preparations are placed in contact with non-aqueous solutions of the substrates. The organic solvents used were either soluble, such as acetone, or practically insoluble in water, such as benzene, carbon tetrachloride and others.

The active esterase preparations used in the majority of the experiments were obtained from pig pancreas.

Prep. A (tissue pulp) was obtained by passing the fat-free pancreas three times through a mincing machine and triturating the product in a mortar.

The dry prep. B was obtained by shaking tissue pulp for 1 hour with five volumes of acetone, decanting the supernatant liquid and repeating the process. After squeezing out the solvent the preparation was spread on filter-paper and air-dried. The dry preparation was then ground in a coffee mill; the final product contained 8–12 % of water.

The activity of this preparation depends in great measure on the water content. When this is low, an approximate proportionality may be found between the water content and the velocity of reactions catalysed by the preparation [Sym, 1933, 1].

In the case of prep. C the aqueous extract of the esterase was obtained by shaking the dry preparation described above with 5 volumes of water for 10 min., centrifuging and passing the centrifugate repeatedly through a layer of infusorial earth. The activity of the clear extracts obtained was studied after adding 10 g. of sodium butyrate or acetate to 100 ml. of extract.

Prep. D was obtained by evaporating prep. C (without sodium salt) in flasks at 20 mm. pressure until the residue had the consistency of a compact gel adherent to the bottom of the flask. The gelatinous mass so obtained is more resistant to inactivating factors than the extract from which it originated and it may be used repeatedly.

The above preparations exhibit esterification activity only in the presence of water. Thus preps. A, B and D are reversibly inactivated by further dehydration, being reactivated by addition of water.

Systems containing organic solvents insoluble in water, such as benzene, can, as in the case of prep. C, be of varied water content without the activity being destroyed. But when water-soluble organic solvents are used, the enzyme undergoes irreversible inactivation in presence of higher concentrations of water [Sym, 1933, 1].

The action of the enzymic preparations was studied in most cases in systems containing *n*-butyl alcohol (*M* BuOH), 0·43 *M* butyric acid and benzene.

The velocity of the reactions was studied by determining the initial velocity of esterification, *v*, *viz.* the number of millimols. of ester per litre which is formed in the non-aqueous phase during the first hour of reaction.

5 ml. of the benzene layer were titrated with 0·1 N alcoholic NaOH using *o*-cresolphthalein as indicator. The reactions were conducted in a Köhler water thermostat with constant shaking and mostly carried out with two samples. Determinations of acidity were made before the reaction started with 5 ml. of each of the two samples. After some time a second determination was made for one of the samples and some time later one for the second sample.

The following considerations illustrate the mechanism of reactions in systems of the type described. After addition of the benzene solution to the aqueous enzyme preparation the substrates dissolved in the benzene diffuse into the aqueous phase where they enter into reaction under the influence of the enzyme. The resultant products are then partitioned between the aqueous and the non-aqueous phases. With the conclusion of the reaction, two equilibria are established: an equilibrium of reaction in the aqueous phase and an equilibrium of distribution of the substrates and of the products of reaction between the aqueous and the non-aqueous phases. These equilibria may be represented as follows:

$$\begin{array}{lcccc} \text{non-aqueous phase:[acid]} & & \text{[alcohol]} & & \text{[ester]} \quad [H_2O] \\ \qquad\qquad\qquad\qquad\quad \downarrow\uparrow & & \downarrow\uparrow & & \downarrow\uparrow \\ \text{aqueous phase} \quad \text{:[acid]} & + & \text{[alcohol]} & \rightleftharpoons & \text{[ester]} + [H_2O] \end{array}$$

The application of organic solvents practically insoluble in water gives much higher yields of esters (often over 95 %) than the use of aqueous systems owing to the fact that the products of reaction are removed from the medium where the reaction proceeds. The reaction of hydrolysis of esters can be investigated by using water-soluble organic solvents with a certain limited content of water (such as acetone, pyridine *etc.*).

In view of the fact that the volume of non-aqueous phase taken was large in comparison with that of the aqueous phase and that the partition coefficients of the substrates and reaction products were such as to favour greater concentration in the non-aqueous phase, the latter may be regarded as a reservoir of the components of the reaction supplying substrates to the aqueous phase and removing esters from it. The progress of the reaction is conveniently studied in the non-aqueous phase without the necessity of removing enzyme from the system.

Preliminary experiments.

The velocity of the reaction rises rapidly with increasing comminution of the pancreas tissue, whilst for prep. B the differences in the size of the grains do not influence the value of v. In shaken systems of preps. A and C, the reaction proceeds about twice as quickly as in unshaken systems. Shaking and the magnitude of interfaces have no influence on the velocity of systems in which prep. D is present.

The results of experiments on the effect of varying the relative amounts of preps. A, B, and C with and without shaking are given in Table I.

The initial velocity of reaction was in all cases found to be proportional to the quantity of prep. B taken whilst in the case of prep. A this proportionality was observed only in systems containing 1 g. and less of preparation.

In the case of prep. C, v is proportional to the volume of preparation taken for shaken systems containing 10 ml. of extract but not for unshaken ones.

Finally the effect of varying the amount of prep. D was examined by evaporating 7 ml. of extract C in one flask and 14 ml. in another, to yield films of about 35 sq. cm. in area; 12·5 ml. of 2 M BuOH and 0·43 M butyric acid in benzene were added to each flask. The values of v found were 7·9 and 19·1 millimols. per litre per hour, respectively (at 37·1°), whence it follows that the

Table I. *Influence of varying the relative amounts of esterase on the velocity of reaction.*

The systems contain 20 ml. of 0·43 *M* butyric acid and *M* BuOH in benzene. Temperature 37°. The systems were shaken.

Amount of preparation g.	*v* With pulp (A) In system	*v* With pulp (A) Per g.	*v* With dry prep. (B) In system	*v* With dry prep. (B) Per g.
0·25	16·2	64·8	15·2	60·8
0·5	29·3	58·6	36·4	72·8
1·0	62·6	62·6	77·7	77·7
2·0	89·9	44·9	149·5	74·8
4·0	116·0	29·0	—	—

The systems contain 50 ml. of benzene solution of 0·43 *M* butyric acid, *M* BuOH, and 2·5–20 ml. of extract C. Temperature 37°.

Vol. of extract taken ml.	*v* Unshaken systems In system	*v* Unshaken systems Per ml. of extract	*v* Shaken systems In system	*v* Shaken systems Per ml. of extract
2·5	2·9	11·6	3·9	15·6
5·0	5·2	10·4	8·1	16·2
10·0	5·9	5·9	15·2	15·2
20·0	7·8	3·9	23·3	11·7

velocity of reaction was greatly dependent on the thickness of the films. In another case, in which both substrates were practically insoluble in water, 75 ml. of 0·4 *M* oleic acid and 0·3 *M* cetyl alcohol in benzene were added to the films prepared from 15 and 30 ml. of prep. C; the values of *v* found were 1·9 and 2·9 m.mols. per litre per hour.

It must be accepted that all substrates, whether readily or difficultly soluble in water, react in the aqueous phase. It is possible that the aqueous phase contains some substance which enhances the solubility of practically insoluble substrates such as cetyl alcohol and oleic acid; it will be shown in a future paper that the presence of bile acids and soap, which form soluble complexes with such substrates, greatly accelerates reaction in systems containing two liquid layers.

The author expressed the opinion in a previous paper [1930] that the reaction between oleic acid and glycerol took place at the phase-boundary, at which a pellicle of esterase formed. This opinion should now be modified: the reaction takes place in the thickness of the pellicle formed.

It has now been found that reversible inactivation commences when the water content in preps. A and D falls below 25 % and is well marked in preparations of the type of A, C and D, dried in a high vacuum at 50° to a water content of 5 %. This supports the view that the reaction takes place in the water of imbibition of the esterase; dehydration beyond a certain point leads to breaks in the continuity of this medium, thereby rendering impossible diffusion of substrates to, and of reaction products from, the enzymic surface.

The activity of 2 g. of prep. B in a system consisting of 25 ml. of *M* BuOH and 0·43 *M* butyric acid in CCl_4 at $-20°$ was found to be quite considerable (about 50 % of ester after 24 hours); a second identical system to which water had been added was inactivated in these conditions. The conclusion might be drawn that the water of imbibition remains liquid at $-20°$, whilst in the second system, containing free water, a layer of ice forms in which diffusion cannot take place.

The addition of salts such as sodium acetate, butyrate, oleate or carbonate and aqueous ammonia prevents excessive acidification and hence greatly accelerates reaction in the presence of preps. A, B and D, and is a condition of the activity of prep. C. The results of experiments illustrating these points are given in Table II.

Table II. *Accelerating action of Na acetate and butyrate.*

Systems contain 20 ml. of *M* BuOH and 0·43 *M* butyric acid in benzene, 1·5 g. of prep. A, or 0·5 g. of prep. B and Na acetate or butyrate. Acceleration is expressed as % activation as compared with systems without salts.

Amount of salt added	% activation			
	By Na acetate		By Na butyrate	
g.	Prep. A	Prep. B	Prep. A	Prep. B
0·05	—	34	—	46
0·1	—	35	—	60
0·15	50	—	111	—
0·2	—	25	—	43
0·3	44	—	96	—
0·4	—	6	—	5
0·6	47	—	Inactivation	—
0·8	—	—	—	Inactivation
1·2	6	—	Inactivation	—
2·4	Inactivation	—	,,	—

Influence of concentration of substrates on the velocity of enzymatic esterification.

It has been shown [Sym, 1931; 1933, 1, 2] that in solvents soluble in water such as acetone the value of the velocity coefficient rises continuously with increase in concentration of one or both of the substrates (except in those cases in which increase in concentration of the acid leads to inactivation of the enzyme). In the case of solvents not soluble in water (benzene, CCl_4) progressive increase in the concentration of alcohols leads to a maximum value of v at a certain concentration, above which the value of v falls; increasing the concentration of acid, on the other hand, leads to a continuous increase in v.

The results (Table III) show that v is roughly doubled by raising the concentration of acid from 0·215 to 0·43 *M*; increasing the concentration to 0·86 *M*

Table III. *Influence of varying the concentration of butyric acid on the velocity of esterification with BuOH.*

Systems contain 20 ml. of *M* BuOH and 0·215–1·72 *M* butyric acid in benzene, with preps. A, B, C, and Na acetate. The values are given in brackets in those cases in which gradual inactivation of the enzyme took place.

	1 g. of prep. A with 0·07 g. of Na acetate		1 g. of prep. B with 0·1 g. of Na acetate		5 ml. of prep. C with 0·5 g. of Na acetate	
Initial conc. of butyric acid *M*	v m.mol.	Concn. of ester after 48 hours m.mol.	v m.mol.	Concn. of ester after 48 hours m.mol.	v m.mol.	Concn. of ester after 72 hours m.mol.
0·215	72	210	390	208	6·6	188
0·43	112	361	686	408	11·9	386
0·86	118	684	695	794	17·0	(378)
1·72	Rapid inactivation	—	(330)	(919)	Rapid inactivation	—

does not to any great extent further increase the value of v, whilst concentrations of 1·72 M in all cases inactivated the enzyme.

The effect of varying the concentration of alcohol is given in Table IV, from which it appears that maximum values of v are obtained for preps. A, B, C and

Table IV. *Influence of varying the concentration of BuOH on the velocity of esterification with butyric acid.*

Systems contain 20 ml. of 0·25–8 M BuOH and 0·43 M butyric acid in benzene, with preps. A, B, C and D. Temperature 37°.

Initial concn. of BuOH M	2·5 g. of prep. A		1 g. of prep. B		5 ml. of prep. C with 0·5 g. of Na butyrate		Prep. D from 20 ml. of prep. C	
	v m.mol.	Concn. of ester after 45 hours m.mol. /litre	v m.mol.	Concn. of ester after 45 hours m.mol. /litre	v m.mol.	Concn. of ester after 120 hours m.mol. /litre	v m.mol.	Concn. of ester after 216 hours m.mol. /litre
0·25	44	181	132	220	1·9	189	9·9	250
0·5	67	322	202	380	3·3	345	14·4	404
1·0	138	382	240	412	4·7	386	23·2	414
2·0	143	372	200	412	5·2	386	19·4	402
4·0	129	366	129	386	3·6	351	16·2	402
8·0	79	259	109	367	2·0	215	—	—

D in presence of 1–2 M BuOH. This phenomenon is, as will be seen later, partly ascribable to variations in the partition coefficients of the substrates between the aqueous and non-aqueous phases due to changes in the concentration of BuOH.

Velocity of esterification of different alcohols and acids, varying the concentration of alcohols.

The results given in Table V indicate that for different alcohols maximum values for v are obtained when molar concentrations are taken and that in general, with the exception of *iso*propyl alcohol, the velocity of esterification falls with increase in the molecular weight of the alcohol.

Table V. *Effect of varying the concentration of different alcohols on the velocity of esterification with butyric acid.*

Systems contain 20 ml. of 0·43 M butyric acid and 0·125–4 M alcohol in benzene or 20 ml. of 0·43 M butyric acid in alcohol, and 1 g. of prep. B. Temperature 37°. Final concentration of ester determined after 7 days.

Initial concn. of alcohol M	*n*-Propyl		*iso*Propyl		*n*-Butyl		*iso*Amyl		*n*-Octyl		Cetyl alcohol	
	v m.mol.	Concn. of ester m.mol. /litre	v m.mol.	Concn. of ester m.mol. /litre	v m.mol.	Concn. of ester m.mol. /litre	v m.mol.	Concn. of ester m.mol. /litre	v m.mol.	Concn. of ester m.mol. /litre	v m.mol.	Concn. of ester m.mol. /litre
0·125	146	—	2·7	114	58	—	76	120	55	123	19	120
0·25	204	—	4·6	209	90	230	86	240	76	240	31	240
0·5	284	384	5·9	301	141	390	99	406	92	394	51	408
1·0	316	398	8·2	373	154	410	126	416	104	416	57	410
2·0	180	396	7·0	327	135	402	101	396	59	400	52	382
4·0	152	—	6·2	262	79	398	79	394	—	—	—	—
Alcohol taken as solvent	Inactivation		—	—	35	394	44	390	14	388	92(?)	396

The effect of varying the concentration of BuOH was examined for a number of acids. The results given in Table VI indicate that in benzene the velocity of reaction falls with increasing molecular weight of the acids and that the highest values of v are obtained also with molar concentrations with the exception of stearic acid.

Table VI. *Effect of varying concentration of BuOH on the velocity of esterification with different acids.*

Systems contain 20 ml. of 0·43 *M* acid and 0·125–4 *M* BuOH in benzene or 20 ml. of 0·43 *M* acid in BuOH and 1 g. of prep. B. Temperature 37°.

	n-Butyric		*n*-Hexanoic		Lauric		Stearic acid	
Concn. of BuOH *M*	*v* m.mol.	Concn. of ester after 168 hours m.mol. /litre	*v* m.mol.	Concn. of ester after 168 hours m.mol. /litre	*v* m.mol.	Concn. of ester after 168 hours m.mol. /litre	*v* m.mol.	Concn. of ester after 168 hours m.mol. /litre
0·125	32	124	29	123	—	—	—	—
0·25	50	248	41	210	15	223	5	232
0·5	75	398	53	380	26	293	9	292
1·0	82	412	62	390	40	400	13	398
2·0	70	—	48	384	40	392	16	390
4·0	44	394	40	—	34	380	19	392
BuOH as solvent	15	394	31	354	28	350	22	406

Optimum conditions of determining the esterase activity of various preparations.

On the basis of the above experiments, the following standard procedure is recommended for various preparations. The pig pancreas is treated as for prep. A above. 1 g. portions of pulp are added to 25 ml. of 0·5–0·1 *M* BuOH and 0·43 *M* butyric acid in benzene, and v is determined after 30 and 60 min.

In the case of other tissues (liver, stomach, intestines), 5 g. of pulp should be taken, the concentration of butyric acid should not exceed 0·1–0·2 *M*, and that of BuOH should be 1·0 *M* in 25 ml. benzene solution. Addition of 0·5 g. of Na acetate is necessary for these tissues. The systems should be very vigorously shaken, and the second titration should be made after 12–24 hours at 37°.

When preparations of the type of prep. B of pancreas are taken, the systems should consist of 0·5 g. of preparation with 25 ml. of benzene solution as for prep. A. The second titration should be performed after 30–60 min.

Aqueous or 20 % glycerol extracts of prep. B should always contain 10 % of sodium acetate or butyrate. The systems should consist of 5 ml. of extract and 25 ml. of benzene solution as for prep. A. The second titration should be made after 5–6 hours.

Films of the type of prep. D are obtained from 10 ml. of aqueous extract. 12·5 ml. of benzene solution as for prep. A are added, 2·5 ml. being taken for titration; the second titration is performed after 3–4 hours.

When it is desired to compare the activities of preparations of the type B care should be taken that the water content of these preparations is not too low.

Partition of substrates between water and benzene taken in various proportions.

The partition of butyric acid and BuOH between benzene and water in presence of different concentrations of alcohols was determined with the object of elucidating the influence of the concentration of the substrate on v. It is

realised that the solvent power of water for butyric acid and BuOH in systems containing enzyme preparations may differ from that of pure water, but the difference is probably not great.

The results, given in Table VII, indicate that the concentration of acid in the aqueous phase in all cases falls with increasing concentration of alcohol.

Table VII. *Partition coefficients of butyric acid between benzene and water in presence of different concentrations of alcohols.*

Systems consist of 50 ml. of 0–8·0 *M* alcohol and 0·43 *M* butyric acid as initial concentration in benzene, and 10 ml. of water. Temperature 14·5°.

Concn. of alcohol	*n*-Propyl	*n*-Butyl	*n*-Octyl	Cetyl	*iso*Propyl alcohol
0	1·80	1·80	1·80	1·80	1·80
0·25	—	2·23	2·30	2·38	—
0·5	2·27	2·44	2·57	2·60	2·20
1·0	2·92	3·02	3·48	3·41	2·67
2·0	4·06	4·35	5·02	—	3·80
4·0	6·47	6·40	—	—	6·0
8·0	8·0	7·5	—	—	—

The values for alcohols practically insoluble in water are fairly close to each other, whilst those for lower alcohols are on the whole lower. It would appear from these results that the values of the partition coefficients are independent of the number of C atoms in the alcohol but are in some way related to solubility of the alcohol in water.

The partition coefficients at 18°, determined in systems containing 10 ml. of water and 50 ml. of benzene in which 0·215, 0·43, 0·86, and 1·72 *M* butyric acid and *M* BuOH were dissolved, were respectively 3·1, 3·1, 3·8 and 4·5 showing that the concentration of acid in the aqueous phase on the whole rises with increasing concentration in benzene. Thus the rise of v with the rise in the concentration of acid is explained.

The concentration of butyric acid in water is lowered by addition of sodium butyrate as was shown by determining the partition coefficient of the acid. It follows that the activating effect of sodium butyrate must be due chiefly to its influence on the acidity of the aqueous phase. Thus the p_H of the aqueous phase of the system, 50 ml. of 0·43 *M* butyric acid and *M* BuOH in benzene + 10 ml. of water, was 3·4 as compared with 5·9 when the aqueous phase contained 1 g. of sodium acetate.

The concentration of BuOH in the aqueous phase after the attainment of equilibrium between the benzene and the aqueous phase was determined in the following manner. 50 ml. of a solution of 0·43 *M* butyric acid and of BuOH in various concentrations in benzene were shaken for 15 min. with 10 ml. of water. After 24 hours 4 g. of anhydrous K_2CO_3 were dissolved in 5 ml. taken from the aqueous layer. This solution was shaken with 10 ml. of *i*-amyl ether, 5 ml. of the resulting solution were taken and 1·6 g. of CaO added. After 24 hours the content of BuOH in the dried solution of *i*-amyl ether solution was determined by means of the method of Tschugaeff and Zerewitinoff [Houben-Weyl, 1930]. For the initial concentrations of BuOH in benzene, 0·25, 0·5, 1·0 and 4·0 *M*, the following concentrations of BuOH in water were noted: 0·075, 0·18, 0·29, 0·45 and 0·55 *M* respectively (temperature: 14·5°).

On the basis of the above data on the partitions of substrates it is now possible to attempt to solve the problem whether there is some connection

between v and the concentration of the substrates in the aqueous phase. From the theoretical point of view the following equation can be expected:

$$v = k\,[\text{butyric acid}]\,[\text{BuOH}].$$

50 ml. of water were added to 15 g. of prep. C. After removing the larger particles of tissue from the mixture by centrifuging, 5 ml. of the turbid centrifugate were taken for reaction. Such an aqueous phase containing esterase in the form of suspended particles of prep. B does not need sodium acetate for the maintenance of activity. Table VIII gives the results of these experiments.

Table VIII. *Dependence between the concentration of substrates in the aqueous phase and the velocity of enzymic esterification.*

25 ml. benzene solution of 0·43 *M* butyric acid and BuOH in various concentrations (5 ml. of the benzene solution taken for the first titration), 5 ml. of enzyme preparation. Temperature 14·5°.

Concn. of BuOH in benzene *M*	Concn. of butyric acid in water *M*	Concn. of BuOH in water *M*	*v* m.mol.	*v*/[acid] [BuOH] = *k*
0·25	0·164	0·075	0·029	2·4
0·5	0·132	0·18	0·042	1·8
1·0	0·095	0·29	0·055	2·0
2·0	0·064	0·45	0·054	1·9
4·0	0·054	0·55	0·044	1·5

The values of k obtained indicate that the dependence of v on concentrations of BuOH in the systems studied is primarily influenced by the concentrations of substrates in the aqueous phase.

Application of the method of Tschugaeff and Zerewitinoff to the determination of enzymic esterification.

In the method described for the examination of esterase, enzymic esterification can be followed not only by determining the decrease in acidity but also by determining the drop in alcohol concentration. The method of Tschugaeff and Zerewitinoff can be applied for the latter determinations as the following experiment proves.

50 ml. of a benzene solution containing 0·5 *M* BuOH and 0·5 *M* butyric acid were subjected to esterification by means of 2 g. of prep. B. The temperature of reaction was 37°. The determination of acidity was carried out as above. The determination of the drop in alcohol concentration was effected by adding 2·5 g. of anhydrous K_2CO_3 to 5 ml. of the filtered benzene solution in order to remove the butyric acid. After shaking and centrifuging, 1 ml. was taken from the upper layer of the solution for determination of hydroxyl groups. It was found that after 1 hour the concentration of butyric acid dropped by 84 millimols./litre. The drop in the concentration of BuOH during the same time was 98 millimols./litre. After 2 hours, the drop in acid concentration was 195 millimols./litre and that in alcohol concentration 210 millimols./litre. Thus it can be seen that there are certain deviations, but the method described can undoubtedly be further improved.

Summary.

1. A method for the determination of the activity of esterase preparations has been described depending on the addition to the given preparations of solutions in organic solvents of the substrates. The degree of esterification can

be determined in the solvent phase on the basis of the drop in acidity and alcohol concentration.

2. The velocity of reaction v rises with increasing concentration of acid, whilst when the concentration of alcohol is raised the velocity of reaction rises to a maximum at molar concentration of alcohol, thereafter falling.

3. This influence of concentrations of butyric acid and BuOH on v has been explained to some extent by the aid of coefficients of partition of substrates of esterification between the benzene and the aqueous phase.

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